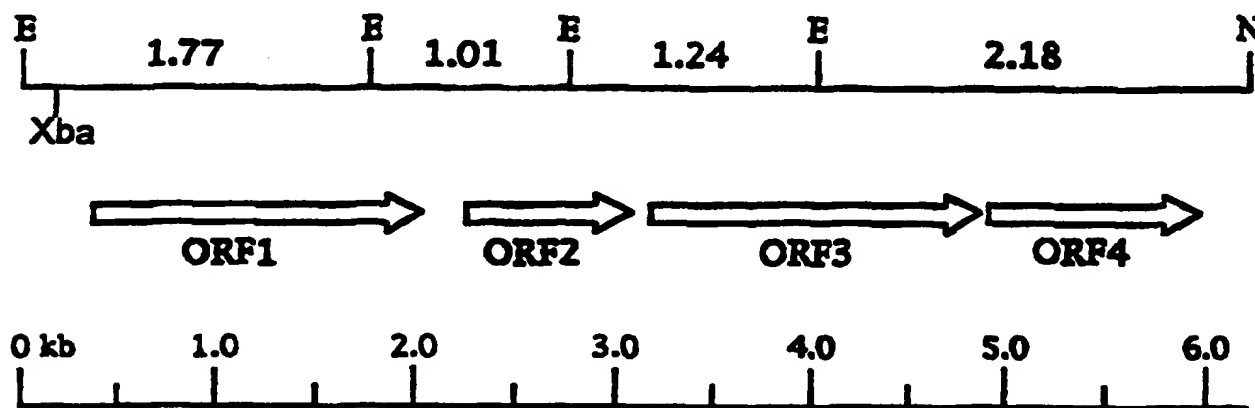




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N		A2	(11) International Publication Number: WO 95/33818
			(43) International Publication Date: 14 December 1995 (14.12.95)
(21) International Application Number: PCT/IB95/00414		(US). LAM, Stephen, Ting [US/US]; 8900 Jeannew Court, Raleigh, NC 27613 (US). HAMMER, Philip, Eugene [US/US]; 212 A Waldo Street, Cary, NC 27511 (US). UKNES, Scott, Joseph [US/US]; 1003 Pinedale Drive, Apex, NC 27502 (US).	
(22) International Filing Date: 30 May 1995 (30.05.95)		(74) Common Representative: CIBA-GEIGY AG; Patentabteilung, Klybeckstrasse 141, CH-4002 Basle (CH).	
(30) Priority Data: 08/258,261 8 June 1994 (08.06.94) US		(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TM, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).	
(60) Parent Application or Grant (63) Related by Continuation US 08/258,261 (CIP) Filed on 8 June 1994 (08.06.94)		Published Without international search report and to be republished upon receipt of that report.	
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(54) Title: GENES FOR THE SYNTHESIS OF ANTIPATHOGENIC SUBSTANCES

Prn Gene Region of MOCG134

(57) Abstract

The present invention is directed to the production of an antipathogenic substance (APS) in a host via recombinant expression of the polypeptides needed to biologically synthesize the APS. Genes encoding polypeptides necessary to produce particular antipathogenic substances are provided, along with methods for identifying and isolating genes needed to recombinantly biosynthesize any desired APS. The cloned genes may be transformed and expressed in a desired host organisms to produce the APS according to the invention for a variety of purposes, including protecting the host from a pathogen, developing the host as a biocontrol agent, and producing large uniform amounts of the APS.

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GENES FOR THE SYNTHESIS OF ANTIPATHOGENIC SUBSTANCES

The present invention relates generally to the protection of host organisms against pathogens, and more particularly to the protection of plants against phytopathogens. In one aspect it provides transgenic plants which have enhanced resistance to phytopathogens and biocontrol organisms with enhanced biocontrol properties. It further provides methods for protecting plants against phytopathogens and methods for the production of antipathogenic substances.

Plants routinely become infected by fungi and bacteria, and many microbial species have evolved to utilize the different niches provided by the growing plant. Some phytopathogens have evolved to infect foliar surfaces and are spread through the air, from plant-to-plant contact or by various vectors, whereas other phytopathogens are soil-borne and preferentially infect roots and newly germinated seedlings. In addition to infection by fungi and bacteria, many plant diseases are caused by nematodes which are soil-borne and infect roots, typically causing serious damage when the same crop species is cultivated for successive years on the same area of ground.

Plant diseases cause considerable crop loss from year to year resulting both in economic hardship to farmers and nutritional deprivation for local populations in many parts of the world. The widespread use of fungicides has provided considerable security against phytopathogen attack, but despite \$1 billion worth of expenditure on fungicides, worldwide crop losses amounted to approximately 10% of crop value in 1981 (James, Seed Sci. & Technol. 9: 679-685 (1981)). The severity of the destructive process of disease depends on the aggressiveness of the phytopathogen and the response of the host, and one aim of most plant breeding programs is to increase the resistance of host plants to disease. Novel gene sources and combinations developed for resistance to disease have typically only had a limited period of successful use in many crop-pathogen systems due to the rapid evolution of phytopathogens to overcome resistance genes. In addition, there are several documented cases of the evolution of fungal strains which are resistant to particular fungicides. As early as 1981, Fletcher and Wolfe (Proc. 1981 Brit. Crop Prot. Conf. (1981))

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contended that 24% of the powdery mildew populations from spring barley, and 53% from winter barley showed considerable variation in response to the fungicide triadimenol and that the distribution of these populations varied between barley varieties with the most susceptible variety also giving the highest incidence of less susceptible fungal types. Similar variation in the sensitivity of fungi to fungicides has been documented for wheat mildew (also to triadimenol), *Botrytis* (to benomyl), *Pyrenophora* (to organomercury), *Pseudocercospora* (to MBC-type fungicides) and *Mycosphaerella fijiensis* to triazoles to mention just a few (Jones and Clifford; Cereal Diseases, John Wiley, 1983). Diseases caused by nematodes have also been controlled successfully by pesticide application. Whereas most fungicides are relatively harmless to mammals and the problems with their use lie in the development of resistance in target fungi, the major problem associated with the use of nematicides is their relatively high toxicity to mammals. Most nematicides used to control soil nematodes are of the carbamate, organochlorine or organophosphorous groups and must be applied to the soil with particular care.

In some crop species, the use of biocontrol organisms has been developed as a further alternative to protect crops. Biocontrol organisms have the advantage of being able to colonize and protect parts of the plant inaccessible to conventional fungicides. This practice developed from the recognition that crops grown in some soils are naturally resistant to certain fungal phytopathogens and that the suppressive nature of these soils is lost by autoclaving. Furthermore, it was recognized that soils which are conducive to the development of certain diseases could be rendered suppressive by the addition of small quantities of soil from a suppressive field (Scher *et al.* Phytopathology 70: 412-417 (1980). Subsequent research demonstrated that root colonizing bacteria were responsible for this phenomenon, now known as biological disease control (Baker *et al.* Biological Control of Plant Pathogens, Freeman Press, San Francisco, 1974). In many cases, the most efficient strains of biological disease controlling bacteria are of the species *Pseudomonas fluorescens* (Weller *et al.* Phytopathology 73: 463-469 (1983); Kloepper *et al.* Phytopathology 71: 1020-1024 (1981)). Important plant pathogens that have been effectively controlled by seed inoculation with these bacteria include *Gaeumannomyces graminis*, the causative agent of take-all in wheat (Cook *et al.* Soil Biol. Biochem 8: 269-273 (1976)) and the *Pythium* and *Rhizoctonia* phytopathogens involved in damping off of cotton (Howell *et al.* Phytopathology 69: 480-482 (1979)). Several biological disease controlling

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Pseudomonas strains produce antibiotics which inhibit the growth of fungal phytopathogens (Howell *et al.* *Phytopathology* 69: 480-482 (1979); Howell *et al.* *Phytopathology* 70: 712-715 (1980)) and these have been implicated in the control of fungal phytopathogens in the rhizosphere. Although biocontrol was initially believed to have considerable promise as a method of widespread application for disease control, it has found application mainly in the environment of glasshouse crops where its utility in controlling soil-borne phytopathogens is best suited for success. Large scale field application of naturally occurring microorganisms has not proven possible due to constraints of microorganism production (they are often slow growing), distribution (they are often short lived) and cost (the result of both these problems). In addition, the success of biocontrol approaches is also largely limited by the identification of naturally occurring strains which may have a limited spectrum of efficacy. Some initial approaches have also been taken to control nematode phytopathogens using biocontrol organisms. Although these approaches are still exploratory, some *Streptomyces* species have been reported to control the root knot nematode (*Meloidogyne* spp.) (WO 93/18135 to Research Corporation Technology), and toxins from some *Bacillus thuringiensis* strains (such as *israeliensis*) have been shown to have broad anti-nematode activity and spore or bacillus preparations may thus provide suitable biocontrol opportunities (EP 0 352 052 to Mycogen, WO 93/19604 to Research Corporation Technologies).

The traditional methods of protecting crops against disease, including plant breeding for disease resistance, the continued development of fungicides, and more recently, the identification of biocontrol organisms, have all met with success. It is apparent, however, that scientists must constantly be in search of new methods with which to protect crops against disease. This invention provides novel methods for the protection of plants against phytopathogens.

The present invention reveals the genetic basis for substances produced by particular microorganisms via a multi-gene biosynthetic pathway which have a deleterious effect on the multiplication or growth of plant pathogens. These substances include carbohydrate containing antibiotics such as aminoglycosides, peptide antibiotics, nucleoside derivatives and other heterocyclic antibiotics containing nitrogen and/or oxygen, polyketides, macrocyclic lactones, and quinones.

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The invention provides the entire set of genes required for recombinant production of particular antipathogenic substances in a host organism. It further provides methods for the manipulation of APS gene sequences for their expression in transgenic plants. The transgenic plants thus modified have enhanced resistance to attack by phytopathogens. The invention provides methods for the cellular targeting of APS gene products so as to ensure that the gene products have appropriate spatial localization for the availability of the required substrate/s. Further provided are methods for the enhancement of throughput through the APS metabolic pathway by overexpression and overproduction of genes encoding substrate precursors.

The invention further provides a novel method for the identification and isolation of the genes involved in the biosynthesis of any particular APS in a host organism.

The invention also describes improved biocontrol strains which produce heterologous APSs and which are efficacious in controlling soil-borne and seedling phytopathogens outside the usual range of the host.

Thus, the invention provides methods for disease control. These methods involve the use of transgenic plants expressing APS biosynthetic genes and the use of biocontrol agents expressing APS genes.

The invention further provides methods for the production of APSs in quantities large enough to enable their isolation and use in agricultural formulations. A specific advantage of these production methods is the uniform chirality of the molecules produced; production in transgenic organisms avoids the generation of populations of racemic mixtures, within which some enantiomers may have reduced activity.

DEFINITIONS

As used in the present application, the following terms have the meanings set out below.

Antipathogenic Substance: A substance which requires one or more nonendogenous enzymatic activities foreign to a plant to be produced in a host where it does not naturally occur, which substance has a deleterious effect on the multiplication or growth of a pathogen (i.e. pathogen). By "nonendogenous enzymatic activities" is meant enzymatic

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activities that do not naturally occur in the host where the antipathogenic substance does not naturally occur. A pathogen may be a fungus, bacteria, nematode, virus, viroid, insect or combination thereof, and may be the direct or indirect causal agent of disease in the host organism. An antipathogenic substance can prevent the multiplication or growth of a phytopathogen or can kill a phytopathogen. An antipathogenic substance may be synthesized from a substrate which naturally occurs in the host. Alternatively, an antipathogenic substance may be synthesized from a substrate that is provided to the host along with the necessary nonendogenous enzymatic activities. An antipathogenic substance may be a carbohydrate containing antibiotic, a peptide antibiotic, a heterocyclic antibiotic containing nitrogen, a heterocyclic antibiotic containing oxygen, a heterocyclic antibiotic containing nitrogen and oxygen, a polyketide, a macrocyclic lactone, and a quinone. Antipathogenic substance is abbreviated as "APS" throughout the text of this application.

Anti-phytopathogenic substance: An antipathogenic substance as herein defined which has a deleterious effect on the multiplication or growth of a plant pathogen (i.e. phytopathogen).

Biocontrol agent: An organism which is capable of affecting the growth of a pathogen such that the ability of the pathogen to cause a disease is reduced. Biocontrol agents for plants include microorganisms which are capable of colonizing plants or the rhizosphere. Such biocontrol agents include gram-negative microorganisms such as *Pseudomonas*, *Enterobacter* and *Serratia*, the gram-positive microorganism *Bacillus* and the fungi *Trichoderma* and *Gliocladium*. Organisms may act as biocontrol agents in their native state or when they are genetically engineered according to the invention.

Pathogen: Any organism which causes a deleterious effect on a selected host under appropriate conditions. Within the scope of this invention the term pathogen is intended to include fungi, bacteria, nematodes, viruses, viroids and insects.

Promoter or Regulatory DNA Sequence: An untranslated DNA sequence which assists in, enhances, or otherwise affects the transcription, translation or expression of an associated structural DNA sequence which codes for a protein or other DNA product. The promoter

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DNA sequence is usually located at the 5' end of a translated DNA sequence, typically between 20 and 100 nucleotides from the 5' end of the translation start site.

Coding DNA Sequence: A DNA sequence that is translated in an organism to produce a protein.

Operably Linked to/Associated With: Two DNA sequences which are "associated" or "operably linked" are related physically or functionally. For example, a promoter or regulatory DNA sequence is said to be "associated with" a DNA sequence that codes for an RNA or a protein if the two sequences are operably linked, or situated such that the regulator DNA sequence will affect the expression level of the coding or structural DNA sequence.

Chimeric Construction/Fusion DNA Sequence: A recombinant DNA sequence in which a promoter or regulatory DNA sequence is operably linked to, or associated with, a DNA sequence that codes for an mRNA or which is expressed as a protein, such that the regulator DNA sequence is able to regulate transcription or expression of the associated DNA sequence. The regulator DNA sequence of the chimeric construction is not normally operably linked to the associated DNA sequence as found in nature. The terms "heterologous" or "non-cognate" are used to indicate a recombinant DNA sequence in which the promoter or regulator DNA sequence and the associated DNA sequence are isolated from organisms of different species or genera.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Restriction map of the cosmid clone pCIB169 from *Pseudomonas fluorescens* carrying the pyrrolnitrin biosynthetic gene region. Restriction sites of the enzymes EcoRI, HindIII, KpnI, NotI, SphI, and XbaI as well as nucleotide positions in kbp are indicated.

Figure 2: Functional Map of the Pyrrolnitrin Gene Region of MOCG134 indicating insertion points of 30 independent Tn5 insertions along the length of pCIB169 for the identification of the genes for pyrrolnitrin biosynthesis. EcoRI restriction sites are

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designated with E, NotI sites with N. The effect of a Tn5 insertion on pm production is designated with either + or -, wherein + indicates a pm producer and - a pm non-producer.

Figure 3: Restriction map of the 9.7 kb MOCG134 Pm gene region of clone pCIB169 involved in pyrrolnitrin biosynthesis. EcoRI restriction sites are designated with E, NotI sites with N, and HindIII sites with H. Nucleotide positions are indicated in kbp.

Figure 4: Location of various subclones derived from pCIB169 isolated for sequence determination purposes.

Figure 5: Localization of the four open reading frames (ORFs 1-4) responsible for pyrrolnitrin biosynthesis in strain MOCG134 on the ~6 kb *XbaI/NotI* fragment of pCIB169 comprising the Pm gene region.

Figure 6: Location of the fragments deleted in ORFs 1-4 in the pyrrolnitrin gene cluster of MOCG134. Deleted fragments are indicated as filled boxes.

Figure 7: Restriction map of the cosmid clone p98/1 from *Sorangium cellulosum* carrying the soraphen biosynthetic gene region. The top line depicts the restriction map of p98/1 and shows the position of restriction sites and their distance from the left edge in kilobases. Restriction sites shown include: B, Bam HI; Bg Bg1 II; E, Eco RI; H, Hind III; Pv, Pvu I; Sm, Sma I. The boxes below the restriction map depict the location of the biosynthetic modules. The activity domains within each module are designated as follows: β -ketoacylsynthase (KS), Acyltransferase (AT), Ketoreductase (KR), Acyl Carrier Protein (ACP), Dehydratase (DH), Enoyl reductase (ER), and Thioesterase (TE).

Figure 8: Construction of pCIB132 from pSUP2021.

Figure 9: Restriction endonuclease map of the phenazine biosynthetic gene cluster contained on a 5.7 kb *EcoRI-HindIII* fragment. Orientation and approximate positions of the six open reading frames are presented below the restriction map. ORF1, which is not entirely present within the 5.7 kb fragment, encodes a product with significant homology to plant DAHP synthases. ORF2 (0.65 kb), ORF3 (0.75 kb), and ORF4 (1.15 kb) have domains homologous to isochorismatase, anthranilate synthase large subunit, and anthranilate synthase small subunit, respectively. ORF5 (0.7 kb) demonstrates no homology with database sequences. The ORF6 (0.65 kb) product has end to end homology with the gene encoding pyridoxine 5'-phosphate oxidase in *E. coli*.

BRIEF DESCRIPTION OF THE SEQUENCES IN THE SEQUENCE LISTING

SEQ ID NO:1:Sequence of the Pyrrolnitrin Gene Cluster
 SEQ ID NO:2:Protein sequence for ORF1 of pyrrolnitrin gene cluster
 SEQ ID NO:3:Protein sequence for ORF2 of pyrrolnitrin gene cluster
 SEQ ID NO:4:Protein sequence for ORF3 of pyrrolnitrin gene cluster
 SEQ ID NO:5:Protein sequence for ORF4 of pyrrolnitrin gene cluster
 SEQ ID NO:6:Sequence of the Soraphen Gene Cluster
 SEQ ID NO:7:Sequence of a Plant Consensus Translation Initiator (Clontech)
 SEQ ID NO:8:Sequence of a Plant Consensus Translation Initiator (Joshi)
 SEQ ID NO:9:Sequence of an Oligonucleotide for Use in a Molecular Adaptor
 SEQ ID NO:10:Sequence of an Oligonucleotide for Use in a Molecular Adaptor
 SEQ ID NO:11:Sequence of an Oligonucleotide for Use in a Molecular Adaptor
 SEQ ID NO:12:Sequence of an Oligonucleotide for Use in a Molecular Adaptor
 SEQ ID NO:13:Sequence of an Oligonucleotide for Use in a Molecular Adaptor
 SEQ ID NO:14:Sequence of an Oligonucleotide for Use in a Molecular Adaptor
 SEQ ID NO:15:Oligonucleotide used to change restriction site
 SEQ ID NO:16:Oligonucleotide used to change restriction site
 SEQ ID NO:17:Sequence of the Phenazine Gene Cluster
 SEQ ID NO:18:Protein sequence for phz1 from the phenazine gene cluster
 SEQ ID NO:19:Protein sequence for phz2 from the phenazine gene cluster
 SEQ ID NO:20:Protein sequence for phz3 from the phenazine gene cluster
 SEQ ID NO:21:DNA sequence for phz4 of Phenazine gene cluster
 SEQ ID NO:22:Protein sequence for phz4 from the phenazine gene cluster

DEPOSITS

Clone	Accession Number	Date of Deposit
pJL3	NRRL B-21254	May 20, 1994
p98/1	NRRL B-21255	May 20, 1994
pCIB169	NRRL B-21256	May 20, 1994
pCIB3350	NRRL B-21257	May 20, 1994
pCIB3351	NRRL B-21258	May 20, 1994

Production of Antipathogenic Substances by Microorganisms

Many organisms produce secondary metabolites and some of these inhibit the growth of other organisms. Since the discovery of penicillin, a large number of compounds with antibiotic activity have been identified, and the number continues to increase with ongoing screening efforts. Antibiotically active metabolites comprise a broad range of chemical structures. The most important include: aminoglycosides (*e.g.* streptomycin) and other carbohydrate containing antibiotics, peptide antibiotics (*e.g.* β -lactams, rhizoctin (see Rapp, C. *et al.*, *Liebigs Ann. Chem.* : 655-661 (1988)), nucleoside derivatives (*e.g.* blasticidin S) and other heterocyclic antibiotics containing nitrogen (*e.g.* phenazine and pyrrolnitrin) and/or oxygen, polyketides (*e.g.* soraphen), macrocyclic lactones (*e.g.* erythromycin) and quinones (*e.g.* tetracycline).

Aminoglycosides and Other Carbohydrate Containing Antibiotics

The aminoglycosides are oligosaccharides consisting of an aminocyclohexanol moiety glycosidically linked to other amino sugars. Streptomycin, one of the best studied of the group, is produced by *Streptomyces griseus*. The biochemistry and biosynthesis of this compound is complex (for review see Mansouri *et al.* in: Genetics and Molecular Biology of Industrial Microorganisms (*ed.*: Hershberger *et al.*), American Society for Microbiology, Washington, D. C. pp 61-67 (1989)) and involves 25 to 30 genes, 19 of which have been analyzed so far (Retzlaff *et al.* in: Industrial Microorganisms: Basic and Applied Molecular Genetics (*ed.*: Baltz *et al.*), American Society for Microbiology, Washington, D. C. pp 183-194 (1993)). Streptomycin, and many other aminoglycosides, inhibits protein synthesis in the target organisms.

Peptide Antibiotics

Peptide antibiotics are classifiable into two groups: (1) those which are synthesized by enzyme systems without the participation of the ribosomal apparatus, and (2) those which require the ribosomally-mediated translation of an mRNA to provide the precursor of the antibiotic.

Non-Ribosomal Peptide Antibiotics are assembled by large, multifunctional enzymes which activate, modify, polymerize and in some cases cyclize the subunit amino acids, forming polypeptide chains. Other acids, such as aminoadipic acid, diaminobutyric acid,

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diaminopropionic acid, dihydroxyamino acid, isoserine, dihydroxybenzoic acid, hydroxyisovaleric acid, (4R)-4-[(E)-2-butenyl]-4,N-dimethyl-L-threonine, and ornithine are also incorporated (Katz & Demain, *Bacteriological Review* 41: 449-474 (1977); Kleinkauf & von Dohren, *Annual Review of Microbiology* 41: 259-289 (1987)). The products are not encoded by any mRNA, and ribosomes do not directly participate in their synthesis. Peptide antibiotics synthesized non-ribosomally can in turn be grouped according to their general structures into linear, cyclic, lactone, branched cyclopeptide, and depsipeptide categories (Kleinkauf & von Dohren, *European Journal of Biochemistry* 192: 1-15 (1990)). These different groups of antibiotics are produced by the action of modifying and cyclizing enzymes; the basic scheme of polymerization is common to them all. Non-ribosomally synthesized peptide antibiotics are produced by both bacteria and fungi, and include edeine, linear gramicidin, tyrocidine and gramicidin S from *Bacillus brevis*, mycobacillin from *Bacillus subtilis*, polymyxin from *Bacillus polymyxa*, etamycin from *Streptomyces griseus*, echinomycin from *Streptomyces echinatus*, actinomycin from *Streptomyces clavuligerus*, enterochelin from *Escherichia coli*, gamma-(alpha-L-aminoadipyl)-L-cysteinyl-D-valine (ACV) from *Aspergillus nidulans*, alamethicine from *Trichoderma viride*, destruxin from *Metarhizium anisopliae*, enniatin from *Fusarium oxysporum*, and beauvericin from *Beauveria bassiana*. Extensive functional and structural similarity exists between the prokaryotic and eukaryotic systems, suggesting a common origin for both. The activities of peptide antibiotics are similarly broad, toxic effects of different peptide antibiotics in animals, plants, bacteria, and fungi are known (Hansen, *Annual Review of Microbiology* 47: 535-564 (1993); Katz & Demain, *Bacteriological Reviews* 41: 449-474 (1977); Kleinkauf & von Dohren, *Annual Review of Microbiology* 41: 259-289 (1987); Kleinkauf & von Dohren, *European Journal of Biochemistry* 192: 1-15 (1990); Kolter & Moreno, *Annual Review of Microbiology* 46: 141-163 (1992)).

Ribosomally-Synthesized Peptide Antibiotics are characterized by the existence of a structural gene for the antibiotic itself, which encodes a precursor that is modified by specific enzymes to create the mature molecule. The use of the general protein synthesis apparatus for peptide antibiotic synthesis opens up the possibility for much longer polymers to be made, although these peptide antibiotics are not necessarily very large. In addition to a structural gene, further genes are required for extracellular secretion and immunity, and these genes are believed to be located close to the structural gene, in most cases probably

on the same operon. Two major groups of peptide antibiotics made on ribosomes exist: those which contain the unusual amino acid lanthionine, and those which do not. Lanthionine-containing antibiotics (lantibiotics) are produced by gram-positive bacteria, including species of *Lactococcus*, *Staphylococcus*, *Streptococcus*, *Bacillus*, and *Streptomyces*. Linear lantibiotics (for example, nisin, subtilin, epidermin, and gallidermin), and circular lantibiotics (for example, duramycin and cinnamycin), are known (Hansen, Annual Review of Microbiology 47: 535-564 (1993); Kolter & Moreno, Annual Review of Microbiology 46: 141-163 (1992)). Lantibiotics often contain other characteristic modified residues such as dehydroalanine (DHA) and dehydrobutyrine (DHB), which are derived from the dehydration of serine and threonine, respectively. The reaction of a thiol from cysteine with DHA yields lanthionine, and with DHB yields β -methylanthionine. Peptide antibiotics which do not contain lanthionine may contain other modifications, or they may consist only of the ordinary amino acids used in protein synthesis. Non-lanthionine-containing peptide antibiotics are produced by both gram-positive and gram-negative bacteria, including *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Enterococcus*, and *Escherichia*. Antibiotics in this category include lactacins, lactocins, sakacin A, pediocins, diplococcin, lactococcins, and microcins (Hansen, *supra*; Kolter & Moreno, *supra*).

Nucleoside Derivatives and Other Heterocyclic Antibiotics Containing Nitrogen and/or Oxygen

These compounds all contain heterocyclic rings but are otherwise structurally diverse and, as illustrated in the following examples, have very different biological activities.

Polyoxins and Nikkomycins are nucleoside derivatives and structurally resemble UDP-N-acetylglucosamine, the substrate of chitin synthase. They have been identified as competitive inhibitors of chitin synthase (Gooday, in: Biochemistry of Cell Walls and Membranes in Fungi (ed.: Kuhn *et al.*), Springer-Verlag, Berlin p. 61 (1990)). The polyoxins are produced by *Streptomyces cacaoi* and the Nikkomycins are produced by *S. tendae*.

Phenazines are nitrogen-containing heterocyclic compounds with a common planar aromatic tricyclic structure. Over 50 naturally occurring phenazines have been identified, each differing in the substituent groups on the basic ring structure. This group of compounds are found produced in nature exclusively by bacteria, in particular

Streptomyces, *Sorangium*, and *Pseudomonas* (for review see Turner & Messenger, *Advances in Microbiol Physiology* 27: 211-275 (1986)). Recently, the phenazine biosynthetic genes of a *P. aureofaciens* strain has been isolated (Pierson & Thomashow *MPMI* 5: 330-339 (1992)). Because of their planar aromatic structure, it has been proposed that phenazines may form intercalative complexes with DNA (Hollstein & van Gemert, *Biochemistry* 10: 497 (1971)), and thereby interfere with DNA metabolism. The phenazine myxin was shown to intercalate DNA (Hollstein & Butler, *Biochemistry* 11: 1345 (1972)) and the phenazine lomofungin was shown to inhibit RNA synthesis in yeast (Cannon & Jimenez, *Biochemical Journal* 142: 457 (1974); Ruet *et al.*, *Biochemistry* 14: 4651 (1975)).

Pyrrolnitrin is a phenylpyrrole derivative with strong antibiotic activity and has been shown to inhibit a broad range of fungi (Homma *et al.*, *Soil Biol. Biochem.* 21: 723-728 (1989); Nishida *et al.*, *J. Antibiot.*, ser A, 18: 211-219 (1965)). It was originally isolated from *Pseudomonas pyrocinia* (Arima *et al.*, *J. Antibiot.*, ser. A, 18: 201-204 (1965)), and has since been isolated from several other *Pseudomonas* species and *Myxococcus* species (Gerth *et al.* *J. Antibiot.* 35: 1101-1103 (1982)). The compound has been reported to inhibit fungal respiratory electron transport (Tripathi & Gottlieb, *J. Bacteriol.* 100: 310-318 (1969)) and uncouple oxidative phosphorylation (Lambowitz & Slayman, *J. Bacteriol.* 112: 1020-1022 (1972)). It has also been proposed that pyrrolnitrin causes generalized lipoprotein membrane damage (Nose & Arima, *J. Antibiot.*, ser A, 22: 135-143 (1969); Carlone & Scannerini, *Mycopahtologia et Mycologia Applicata* 53: 111-123 (1974)). Pyrrolnitrin is biosynthesized from tryptophan (Chang *et al.* *J. Antibiot.* 34: 555-566) and the biosynthetic genes from *P. fluorescens* have now been cloned (see Section C of examples). Thus, one embodiment of the present invention relates to an isolated DNA molecule encoding one or more polypeptides for the biosynthesis of pyrrolnitrin in a heterologous host, which molecule can be used to genetically engineer a host organism to express said antipathogenic substance. Other embodiments of the invention are the isolated polypeptides required for the biosynthesis of pyrrolnitrin.

Polyketide Synthases

Many antibiotics, in spite of the apparent structural diversity, share a common pattern of biosynthesis. The molecules are built up from two carbon building blocks, the β -carbon of which always carries a keto group, thus the name polyketide. The tremendous structural

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diversity derives from the different lengths of the polyketide chain and the different side-chains introduced, either as part of the two carbon building blocks, or after the polyketide backbone is formed. The keto groups may also be reduced to hydroxyls or removed altogether. Each round of two carbon addition is carried out by a complex of enzymes called the polyketide synthases (PKS) in a manner similar to fatty acid biosynthesis. The biosynthetic genes for an increasing number of polyketide antibiotics have been isolated and sequenced. It is quite apparent that the PKS genes are structurally conserved. The encoded proteins generally fall into two types: type I proteins are polyfunctional, with several catalytic domains carrying out different enzymatic steps covalently linked together (e.g. PKS for erythromycin, soraphen, and avermectin (Joaua *et al.* Plasmid 28: 157-165 (1992); MacNeil *et al.* in: Industrial Microorganisms: Basic and Applied Molecular Genetics, (ed.: Baltz *et al.*), American Society for Microbiology, Washington D. C. pp. 245-256 (1993)); whereas type II proteins are monofunctional (Hutchinson *et al.* in: Industrial Microorganisms: Basic and Applied Molecular Genetics, (ed.: Baltz *et al.*), American Society for Microbiology, Washington D. C. pp. 203-216 (1993)). For the simpler polyketide antibiotics such as actinorhodin (produced by *Streptomyces coelicolor*), the several rounds of two carbon additions are carried out iteratively on PKS enzymes encoded by one set of PKS genes. In contrast, synthesis of the more complicated compounds such as erythromycin and soraphen (see Section E of examples) involves sets of PKS genes organized into modules, with each module carrying out one round of two carbon addition (for review see Hopwood *et al.* in: Industrial Microorganisms: Basic and Applied Molecular Genetics, (ed.: Baltz *et al.*), American Society for Microbiology, Washington D. C., pp. 267-275 (1993)). The present invention provides the biosynthetic genes of soraphen from *Sorangium* (see Section E of examples). Thus, another embodiment of the present invention relates to an isolated DNA molecule encoding one or more polypeptides for the biosynthesis of soraphen in a heterologous host which molecule can be used to genetically engineer a host organism to express said antipathogenic substance. Other embodiments of the invention are isolated polypeptides required for the biosynthesis of soraphen.

Macrocyclic Lactones

This group of compounds shares the presence of a large lactone ring with various ring substituents. They can be further classified into subgroups, depending on the ring size and other characteristics. The macrolides, for example, contain 12-, 14-, 16-, or 17-membered

lactone rings glycosidically linked to one or more aminosugars and/or deoxysugars. They are inhibitors of protein synthesis, and are particularly effective against gram-positive bacteria. Erythromycin A, a well-studied macrolide produced by *Saccharopolyspora erythraea*, consists of a 14-membered lactone ring linked to two deoxy sugars. Many of the biosynthetic genes have been cloned; all have been located within a 60 kb segment of the *S. erythraea* chromosome. At least 22 closely linked open reading frames have been identified to be likely involved in erythromycin biosynthesis (Donadio *et al.*, in: Industrial Microorganisms: Basic and Applied Molecular Genetics, (ed.: Baltz *et al.*), American Society for Microbiology, Washington D. C., pp 257-265 (1993)).

Quinones

Quinones are aromatic compounds with two carbonyl groups on a fully unsaturated ring. The compounds can be broadly classified into subgroups according to the number of aromatic rings present, *i.e.*, benzoquinones, naphthoquinones, etc. A well studied group is the tetracyclines, which contain a naphthacene ring with different substituents. Tetracyclines are protein synthesis inhibitors and are effective against both gram-positive and gram-negative bacteria, as well as rickettsias, mycoplasma, and spirochetes. The aromatic rings in the tetracyclines are derived from polyketide molecules. Genes involved in the biosynthesis of oxytetracycline (produced by *Streptomyces rimosus*) have been cloned and expressed in *Streptomyces lividans* (Binnie *et al.* J. Bacteriol. 171: 887-895 (1989)). The PKS genes share homology with those for actinorhodin and therefore encode type II (monofunctional) PKS proteins (Hopewood & Sherman, Ann. Rev. Genet. 24: 37-66 (1990)).

Other Types of APS

Several other types of APSs have been identified. One of these is the antibiotic 2-hexyl-5-propyl-resorcinol which is produced by certain strains of *Pseudomonas*. It was first isolated from the *Pseudomonas* strain B-9004 (Kanda *et al.* J. Antibiot. 28: 935-942 (1975)) and is a dialkyl-substituted derivative of 1,3-dihydroxybenzene. It has been shown to have antipathogenic activity against Gram-positive bacteria (in particular *Clavibacter* sp.), mycobacteria, and fungi.

Another type of APS are the methoxyacrylates, such as strobilurin B. Strobilurin B is produced by Basidiomycetes and has a broad spectrum of fungicidal activity (Anke, T. *et*

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al., *Journal of Antibiotics (Tokyo)* 30: 806-810 (1977). In particular, strobilurin B is produced by the fungus *Bolinia lutea*. Strobilurin B appears to have antifungal activity as a result of its ability to inhibit cytochrome b dependent electron transport thereby inhibiting respiration (Becker, W. *et al.*, *FEBS Letters* 132: 329-333 (1981).

Most antibiotics have been isolated from bacteria, actinomycetes, and fungi. Their role in the biology of the host organism is often unknown, but many have been used with great success, both in medicine and agriculture, for the control of microbial pathogens. Antibiotics which have been used in agriculture are: blasticidin S and kasugamycin for the control of rice blast (*Pyricularia oryzae*), validamycin for the control of *Rhizoctonia solani*, prumycin for the control of *Botrytis* and *Sclerotinia* species, and mildiomyacin for the control of mildew.

To date, the use of antibiotics in plant protection has involved the production of the compounds through chemical synthesis or fermentation and application to seeds, plant parts, or soil. This invention describes the identification and isolation of the biosynthetic genes of a number of anti-phytopathogenic substances and further describes the use of these genes to create transgenic plants with enhanced disease resistance characteristics and also the creation of improved biocontrol strains by expression of the isolated genes in organisms which colonize host plants or the rhizosphere. Furthermore, the availability of such genes provides methods for the production of APSs for isolation and application in antipathogenic formulations.

Methods for Cloning Genes for Antipathogenic Substances

Genes encoding antibiotic biosynthetic genes can be cloned using a variety of techniques according to the invention. The simplest procedure for the cloning of APS genes requires the cloning of genomic DNA from an organism identified as producing an APS, and the transfer of the cloned DNA on a suitable plasmid or vector to a host organism which does not produce the APS, followed by the identification of transformed host colonies to which the APS-producing ability has been conferred. Using a technique such as λ ::Tn5 transposon mutagenesis (de Bruijn & Lupski, *Gene* 27: 131-149 (1984)), the exact region of the transforming APS-conferring DNA can be more precisely defined. Alternatively or additionally, the transforming APS-conferring DNA can be cleaved into smaller fragments

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and the smallest which maintains the APS-conferring ability further characterized. Whereas the host organism lacking the ability to produce the APS may be a different species to the organism from which the APS derives, a variation of this technique involves the transformation of host DNA into the same host which has had its APS-producing ability disrupted by mutagenesis. In this method, an APS-producing organism is mutated and non-APS producing mutants isolated, and these are complemented by cloned genomic DNA from the APS producing parent strain. A further example of a standard technique used to clone genes required for APS biosynthesis is the use of transposon mutagenesis to generate mutants of an APS-producing organism which, after mutagenesis, fail to produce the APS. Thus, the region of the host genome responsible for APS production is tagged by the transposon and can be easily recovered and used as a probe to isolate the native genes from the parent strain. APS biosynthetic genes which are required for the synthesis of APSs and which are similar to known APS compounds may be clonable by virtue of their sequence homology to the biosynthetic genes of the known compounds. Techniques suitable for cloning by homology include standard library screening by DNA hybridization.

This invention also describes a novel technique for the isolation of APS biosynthetic genes which may be used to clone the genes for any APS, and is particularly useful for the cloning of APS biosynthetic genes which may be recalcitrant to cloning using any of the above techniques. One reason why such recalcitrance to cloning may exist is that the standard techniques described above (except for cloning by homology) may preferentially lead to the isolation of regulators of APS biosynthesis. Once such a regulator has been identified, however, it can be used using this novel method to isolate the biosynthetic genes under the control of the cloned regulator. In this method, a library of transposon insertion mutants is created in a strain of microorganism which lacks the regulator or has had the regulator gene disabled by conventional gene disruption techniques. The insertion transposon used carries a promoter-less reporter gene (*e.g. lacZ*). Once the insertion library has been made, a functional copy of the regulator gene is transferred to the library of cells (*e.g. by* conjugation or electroporation) and the plated cells are selected for expression of the reporter gene. Cells are assayed before and after transfer of the regulator gene. Colonies which express the reporter gene only in the presence of the regulator gene are insertions adjacent to the promoter of genes regulated by the regulator. Assuming the regulator is specific in its regulation for APS-biosynthetic genes, then the genes tagged by this

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procedure will be APS-biosynthetic genes. In a preferred embodiment, the cloned regulator gene is the *gafA* gene described in PCT application WO 94/01561 which regulates the expression of the biosynthetic genes for pyrrolnitrin. Thus, this method is a preferred method for the cloning of the biosynthetic genes for pyrrolnitrin.

An alternative method for identifying and isolating a gene from a microorganism required for the biosynthesis of an antipathogenic substance (APS), wherein the expression of said gene is under the control of a regulator of the biosynthesis of said APS, comprises

- (a) cloning a library of genetic fragments from said microorganism into a vector adjacent to a promoterless reporter gene in a vector such that expression of said reporter gene can occur only if promoter function is provided by the cloned fragment;
 - (b) transforming the vectors generated from step (a) into a suitable host;
 - (c) identifying those transformants from step (b) which express said reporter gene only in the presence of said regulator; and
 - (d) identifying and isolating the DNA fragment operably linked to the genetic fragment from said microorganism present in the transformants identified in step (c);
- wherein the DNA fragment isolated and identified in step (d) encodes one or more polypeptides required for the biosynthesis of said APS.

In order for the cloned APS genes to be of use in transgenic expression, it is important that all the genes required for synthesis from a particular metabolite be identified and cloned. Using combinations of, or all the techniques described above, this is possible for any known APS. As most APS biosynthetic genes are clustered together in microorganisms, usually encoded by a single operon, the identification of all the genes will be possible from the identification of a single locus in an APS-producing microorganism. In addition, as regulators of APS biosynthetic genes are believed to regulate the whole pathway, then the cloning of the biosynthetic genes via their regulators is a particularly attractive method of cloning these genes. In many cases the regulator will control transcription of the single entire operon, thus facilitating the cloning of genes using this strategy.

Using the methods described in this application, biosynthetic genes for any APS can be cloned from a microorganism. Expression vectors comprising isolated DNA molecules encoding one or more polypeptides for the biosynthesis of an antipathogenic substance

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such as pyrrolnitrin and soraphen can be used to transform a heterologous host. Suitable heterologous hosts are bacteria, fungi, yeast and plants. In a preferred embodiment of the invention the transformed hosts will be able to synthesize an antipathogenic substance not naturally occurring in said host. The host can then be grown under conditions which allow production of said antipathogenic sequence, which can be thus be collected from the host. Using the methods of gene manipulation and transgenic plant production described in this specification, the cloned APS biosynthetic genes can be modified and expressed in transgenic plants. Suitable APS biosynthetic genes include those described at the beginning of this section, viz. aminoglycosides and other carbohydrate containing antibiotics (*e.g.* streptomycin), peptide antibiotics (both non-ribosomally and ribosomally synthesized types), nucleoside derivatives and other heterocyclic antibiotics containing nitrogen and/or oxygen (*e.g.* polyoxins, nikkomycins, phenazines, and pyrrolnitrin), polyketides, macrocyclic lactones and quinones (*e.g.* soraphen, erythromycin and tetracycline). Expression in transgenic plants will be under the control of an appropriate promoter and involves appropriate cellular targeting considering the likely precursors required for the particular APS under consideration. Whereas the invention is intended to include the expression in transgenic plants of any APS gene isolatable by the procedures described in this specification, those which are particularly preferred include pyrrolnitrin, soraphen, phenazine, and the peptide antibiotics gramicidin and epidermin. The cloned biosynthetic genes can also be expressed in soil-borne or plant colonizing organisms for the purpose of conferring and enhancing biocontrol efficacy in these organisms. Particularly preferred APS genes for this purpose are those which encode pyrrolnitrin, soraphen, phenazine, and the peptide antibiotics.

Production of Antipathogenic Substances in Heterologous Microbial Hosts

Cloned APS genes can be expressed in heterologous bacterial or fungal hosts to enable the production of the APS with greater efficiency than might be possible from native hosts. Techniques for these genetic manipulations are specific for the different available hosts and are known in the art. For example, the expression vectors pKK223-3 and pKK223-2 can be used to express heterologous genes in *E. coli*, either in transcriptional or translational fusion, behind the *tac* or *trc* promoter. For the expression of operons encoding multiple ORFs, the simplest procedure is to insert the operon into a vector such as pKK223-3 in transcriptional fusion, allowing the cognate ribosome binding site of the heterologous genes

to be used. Techniques for overexpression in gram-positive species such as *Bacillus* are also known in the art and can be used in the context of this invention (Quax *et al.* *In: Industrial Microorganisms: Basic and Applied Molecular Genetics*, Eds. Baltz *et al.*, American Society for Microbiology, Washington (1993)). Alternate systems for overexpression rely on yeast vectors and include the use of *Pichia*, *Saccharomyces* and *Kluyveromyces* (Sreekrishna, *In: Industrial microorganisms: basic and applied molecular genetics*, Baltz, Hegeman, and Skatrud *eds.*, American Society for Microbiology, Washington (1993); Dequin & Barre, *Biotechnology* 12:173-177 (1994); van den Berg *et al.*, *Biotechnology* 8:135-139 (1990)).

Cloned APS genes can also be expressed in heterologous bacterial and fungal hosts with the aim of increasing the efficacy of biocontrol strains of such bacterial and fungal hosts. Thus, a method for protecting plants against phytopathogens is to treat said plant with a biocontrol agent transformed with one or more vectors collectively capable of expressing all of the polypeptides necessary to produce an anti-pathogenic substance in amounts which inhibit said phytopathogen. Microorganisms which are suitable for the heterologous overexpression of APS genes are all microorganisms which are capable of colonizing plants or the rhizosphere. As such they will be brought into contact with phytopathogenic fungi, bacteria and nematodes causing an inhibition of their growth. These include gram-negative microorganisms such as *Pseudomonas*, *Enterobacter* and *Serratia*, the gram-positive microorganism *Bacillus* and the fungi *Trichoderma* and *Gliocladium*. Particularly preferred heterologous hosts are *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas cepacia*, *Pseudomonas aureofaciens*, *Pseudomonas aurantiaca*, *Enterobacter cloacae*, *Serratia marcescens*, *Bacillus subtilis*, *Bacillus cereus*, *Trichoderma viride*, *Trichoderma harzianum* and *Gliocladium virens*. In preferred embodiments of the invention the biosynthetic genes for pyrrolnitrin, soraphen, phenazine, and/or peptide antibiotics are transferred to the particularly preferred heterologous hosts listed above. In a particularly preferred embodiment, the biosynthetic genes for phenazine and/or soraphen are transferred to and expressed in *Pseudomonas fluorescens* strain CGA267356 (described in the published application EP 0 472 494) which has biocontrol utility due to its production of pyrrolnitrin (but not phenazine). In another preferred embodiment, the biosynthetic genes for pyrrolnitrin and/or soraphen are transferred to *Pseudomonas aureofaciens* strain 30-84 which has biocontrol characteristics due to its production of phenazine. Expression in heterologous biocontrol strains requires the selection of vectors appropriate for replication in

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the chosen host and a suitable choice of promoter. Techniques are well known in the art for expression in gram-negative and gram-positive bacteria and fungi and are described elsewhere in this specification.

Expression of Genes for Anti-phytopathogenic Substances In Plants

A method for protecting plants against phytopathogens is to transform said plant with one or more vectors collectively capable of expressing all of the polypeptides necessary to produce an anti-pathogenic substance in said plant in amounts which inhibit said phythopathogen. The APS biosynthetic genes of this invention when expressed in transgenic plants cause the biosynthesis of the selected APS in the transgenic plants. In this way transgenic plants with enhanced resistance to phytopathogenic fungi, bacteria and nematodes are generated. For their expression in transgenic plants, the APS genes and adjacent sequences may require modification and optimization.

Although in many cases genes from microbial organisms can be expressed in plants at high levels without modification, low expression in transgenic plants may result from APS genes having codons which are not preferred in plants. It is known in the art that all organisms have specific preferences for codon usage, and the APS gene codons can be changed to conform with plant preferences, while maintaining the amino acids encoded. Furthermore, high expression in plants is best achieved from coding sequences which have at least 35% GC content, and preferably more than 45%. Microbial genes which have low GC contents may express poorly in plants due to the existence of ATTTA motifs which may destabilize messages, and AATAAA motifs which may cause inappropriate polyadenylation. In addition, potential APS biosynthetic genes can be screened for the existence of illegitimate splice sites which may cause message truncation. All changes required to be made within the APS coding sequence such as those described above can be made using well known techniques of site directed mutagenesis, PCR, and synthetic gene construction using the methods described in the published patent applications EP 0 385 962 (to Monsanto), EP 0 359 472 (to Lubrizol), and WO 93/07278 (to Ciba-Geigy). The preferred APS biosynthetic genes may be unmodified genes, should these be expressed at high levels in target transgenic plant species, or alternatively may be genes modified by the removal of destabilization and inappropriate polyadenylation motifs and illegitimate splice sites, and further modified by the incorporation of plant preferred codons, and further with a GC content preferred for expression in plants. Although preferred gene sequences may be

adequately expressed in both monocotyledonous and dicotyledonous plant species, sequences can be modified to account for the specific codon preferences and GC content preferences of monocotyledons or dicotyledons as these preferences have been shown to differ (Murray *et al.* Nucl. Acids Res. 17: 477-498 (1989)).

For efficient initiation of translation, sequences adjacent to the initiating methionine may require modification. The sequences cognate to the selected APS genes may initiate translation efficiently in plants, or alternatively may do so inefficiently. In the case that they do so inefficiently, they can be modified by the inclusion of sequences known to be effective in plants. Joshi has suggested an appropriate consensus for plants (NAR 15: 6643-6653 (1987) ; SEQ ID NO:8)) and Clontech suggests a further consensus translation initiator (1993/1994 catalog, page 210; SEQ ID NO:7). These consensus are suitable for use with the APS biosynthetic genes of this invention. The sequences are incorporated into the APS gene construction, up to and including the ATG (whilst leaving the second amino acid of the APS gene unmodified), or alternatively up to and including the GTC subsequent to the ATG (with the possibility of modifying the second amino acid of the transgene).

Expression of APS genes in transgenic plants is behind a promoter shown to be functional in plants. The choice of promoter will vary depending on the temporal and spatial requirements for expression, and also depending on the target species. For the protection of plants against foliar pathogens, expression in leaves is preferred; for the protection of plants against ear pathogens, expression in inflorescences (*e.g.* spikes, panicles, cobs *etc.*) is preferred; for protection of plants against root pathogens, expression in roots is preferred; for protection of seedlings against soil-borne pathogens, expression in roots and/or seedlings is preferred. In many cases, however, expression against more than one type of phytopathogen will be sought, and thus expression in multiple tissues will be desirable. Although many promoters from dicotyledons have been shown to be operational in monocotyledons and *vice versa*, ideally dicotyledonous promoters are selected for expression in dicotyledons, and monocotyledonous promoters for expression in monocotyledons. However, there is no restriction to the provenance of selected promoters; it is sufficient that they are operational in driving the expression of the APS biosynthetic genes. In some cases, expression of APSs in plants may provide protection against insect pests. Transgenic expression of the biosynthetic genes for the APS beauvericin (isolated

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from *Beauveria bassiana*) may, for example provide protection against insect pests of crop plants.

Preferred promoters which are expressed constitutively include the CaMV 35S and 19S promoters, and promoters from genes encoding actin or ubiquitin. Further preferred constitutive promoters are those from the 12(4-28), CP21, CP24, CP38, and CP29 genes whose cDNAs are provided by this invention.

The APS genes of this invention can also be expressed under the regulation of promoters which are chemically regulated. This enables the APS to be synthesized only when the crop plants are treated with the inducing chemicals, and APS biosynthesis subsequently declines. Preferred technology for chemical induction of gene expression is detailed in the published European patent application EP 0 332 104 (to Ciba-Geigy) herein incorporated by reference. A preferred promoter for chemical induction is the tobacco PR-1a promoter.

A preferred category of promoters is that which is wound inducible. Numerous promoters have been described which are expressed at wound sites and also at the sites of phytopathogen infection. These are suitable for the expression of APS genes because APS biosynthesis is turned on by phytopathogen infection and thus the APS only accumulates when infection occurs. Ideally, such a promoter should only be active locally at the sites of infection, and in this way APS only accumulates in cells which need to synthesize the APS to kill the invading phytopathogen. Preferred promoters of this kind include those described by Stanford *et al.* Mol. Gen. Genet. 215: 200-208 (1989), Xu *et al.* Plant Molec. Biol. 22: 573-588 (1993), Logemann *et al.* Plant Cell 1: 151-158 (1989), Rohrmeier & Lehle, Plant Molec. Biol. 22: 783-792 (1993), Firek *et al.* Plant Molec. Biol. 22: 129-142 (1993), and Warner *et al.* Plant J. 3: 191-201 (1993).

Preferred tissue specific expression patterns include green tissue specific, root specific, stem specific, and flower specific. Promoters suitable for expression in green tissue include many which regulate genes involved in photosynthesis and many of these have been cloned from both monocotyledons and dicotyledons. A preferred promoter is the maize PEPC promoter from the phosphoenol carboxylase gene (Hudspeth & Grula, Plant Molec. Biol. 12: 579-589 (1989)). A preferred promoter for root specific expression is that

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described by de Framond (FEBS 290: 103-106 (1991); EP 0 452 269 to Ciba-Geigy) and a further preferred root-specific promoter is that from the T-1 gene provided by this invention. A preferred stem specific promoter is that described in patent application WO 93/07278 (to Ciba-Geigy) and which drives expression of the maize *trpA* gene.

Preferred embodiments of the invention are transgenic plants expressing APS biosynthetic genes in a root-specific fashion. In an especially preferred embodiment of the invention the biosynthetic genes for pyrrolnitrin are expressed behind a root specific promoter to protect transgenic plants against the phytopathogen *Rhizoctonia*. In another especially preferred embodiment of the invention the biosynthetic genes for phenazine are expressed behind a root specific promoter to protect transgenic plants against the phytopathogen *Gaeumannomyces graminis*. Further preferred embodiments are transgenic plants expressing APS biosynthetic genes in a wound-inducible or pathogen infection-inducible manner. For example, a further especially preferred embodiment involves the expression of the biosynthetic genes for soraphen behind a wound-inducible or pathogen-inducible promoter for the control of foliar pathogens.

In addition to the selection of a suitable promoter, constructions for APS expression in plants require an appropriate transcription terminator to be attached downstream of the heterologous APS gene. Several such terminators are available and known in the art (*e.g.* *tm1* from CaMV, E9 from *rbcS*). Any available terminator known to function in plants can be used in the context of this invention.

Numerous other sequences can be incorporated into expression cassettes for APS genes. These include sequences which have been shown to enhance expression such as intron sequences (*e.g.* from *Adh1* and *bronze1*) and viral leader sequences (*e.g.* from TMV, MCMV and AMV).

The overproduction of APSs in plants requires that the APS biosynthetic gene encoding the first step in the pathway will have access to the pathway substrate. For each individual APS and pathway involved, this substrate will likely differ, and so too may its cellular localization in the plant. In many cases the substrate may be localized in the cytosol, whereas in other cases it may be localized in some subcellular organelle. As much biosynthetic activity in the

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plant occurs in the chloroplast, often the substrate may be localized to the chloroplast and consequently the APS biosynthetic gene products for such a pathway are best targeted to the appropriate organelle (*e.g.* the chloroplast). Subcellular localization of transgene encoded enzymes can be undertaken using techniques well known in the art. Typically, the DNA encoding the target peptide from a known organelle-targeted gene product is manipulated and fused upstream of the required APS gene/s. Many such target sequences are known for the chloroplast and their functioning in heterologous constructions has been shown. In a preferred embodiment of this invention the genes for pyrrolnitrin biosynthesis are targeted to the chloroplast because the pathway substrate tryptophan is synthesized in the chloroplast.

In some situations, the overexpression of APS genes may deplete the cellular availability of the substrate for a particular pathway and this may have detrimental effects on the cell. In situations such as this it is desirable to increase the amount of substrate available by the overexpression of genes which encode the enzymes for the biosynthesis of the substrate. In the case of tryptophan (the substrate for pyrrolnitrin biosynthesis) this can be achieved by overexpressing the *trpA* and *trpB* genes as well as anthranilate synthase subunits. Similarly, overexpression of the enzymes for chorismate biosynthesis such as DAHP synthase will be effective in producing the precursor required for phenazine production. A further way of making more substrate available is by the turning off of known pathways which utilize specific substrates (provided this can be done without detrimental side effects). In this manner, the substrate synthesized is channeled towards the biosynthesis of the APS and not towards other compounds.

Vectors suitable for plant transformation are described elsewhere in this specification. For *Agrobacterium*-mediated transformation, binary vectors or vectors carrying at least one T-DNA border sequence are suitable, whereas for direct gene transfer any vector is suitable and linear DNA containing only the construction of interest may be preferred. In the case of direct gene transfer, transformation with a single DNA species or co-transformation can be used (Schocher *et al.* Biotechnology 4: 1093-1096 (1986)). For both direct gene transfer and *Agrobacterium*-mediated transfer, transformation is usually (but not necessarily) undertaken with a selectable marker which may provide resistance to an antibiotic

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(kanamycin, hygromycin or methatrexate) or a herbicide (basta). The choice of selectable marker is not, however, critical to the invention.

Synthesis of an APS in a transgenic plant will frequently require the simultaneous overexpression of multiple genes encoding the APS biosynthetic enzymes. This can be achieved by transforming the individual APS biosynthetic genes into different plant lines individually, and then crossing the resultant lines. Selection and maintenance of lines carrying multiple genes is facilitated if each the various transformation constructions utilize different selectable markers. A line in which all the required APS biosynthetic genes have been pyramided will synthesize the APS, whereas other lines will not. This approach may be suitable for hybrid crops such as maize in which the final hybrid is necessarily a cross between two parents. The maintenance of different inbred lines with different APS genes may also be advantageous in situations where a particular APS pathway may lead to multiple APS products, each of which has a utility. By utilizing different lines carrying different alternative genes for later steps in the pathway to make a hybrid cross with lines carrying all the remaining required genes it is possible to generate different hybrids carrying different selected APSs which may have different utilities.

Alternate methods of producing plant lines carrying multiple genes include the retransformation of existing lines already transformed with an APS gene or APS genes (and selection with a different marker), and also the use of single transformation vectors which carry multiple APS genes, each under appropriate regulatory control (*i.e.* promoter, terminator *etc.*). Given the ease of DNA construction, the manipulation of cloning vectors to carry multiple APS genes is a preferred method.

Before plant propagation material (fruit, tuber, grains, seed) and especially before seed is sold as a commercial product, it is customarily treated with a protectant coating comprising herbicides, insecticides, fungicides, bactericides, nematicides, molluscicides or mixtures of several of these compounds. If desired these compounds are formulated together with further carriers, surfactants or application-promoting adjuvants customarily employed in the art of formulation to provide protection against damage caused by bacterial, fungal or animal pests.

In order to treat the seed, the protectant coating may be applied to the seeds either by impregnating the tubers or grains with a liquid formulation or by coating them with a

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combined wet or dry formulation. In special cases other methods of application to plants are possible such as treatment directed at the buds or the fruit.

A plant seed according to the invention comprises a DNA sequence encoding for the production of an antipathogenic substance and may be treated with a seed protectant coating comprising a seed treatment compound such as captan, carboxin, thiram (TMTD®), methalaxyl (Apron®), pirimiphos-methyl (Actellic®) and others that are commonly used in seed treatment. It is thus a further object of the present invention to provide plant propagation material and especially seed encoding for the production of an antipathogenic substance, which material is treated with a seed protectant coating customarily used in seed treatment.

Production of Antipathogenic Substances in Heterologous Hosts

The present invention also provides methods for obtaining APSs. These APSs may be effective in the inhibition of growth of microbes, particularly phytopathogenic microbes. The APSs can be produced in large quantities from organisms in which the APS genes have been overexpressed, and suitable organisms for this include gram-negative and gram-positive bacteria and yeast, as well as plants. For the purposes of APS production, the significant criteria in the choice of host organism are its ease of manipulation, rapidity of growth (*i.e.* fermentation in the case of microorganisms), and its lack of susceptibility to the APS being overproduced. In a preferred embodiment of the invention enhanced amounts of an antipathogenic substance are synthesized in a host, in which the antipathogenic substance naturally occurs, wherein said host is transformed with one or more DNA molecules collectively encoding the complete set of polypeptides required to synthesize said antipathogenic substance. These methods of APS production have significant advantages over the chemical synthesis technology usually used in the preparation of APSs such as antibiotics. These advantages are the cheaper cost of production, and the ability to synthesize compounds of a preferred biological enantiomer, as opposed to the racemic mixtures inevitably generated by organic synthesis. The ability to produce stereochemically appropriate compounds is particularly important for molecules with many chirally active carbon atoms. APSs produced by heterologous hosts can be used in medical (*i.e.* control of pathogens and/or infectious disease) as well as agricultural applications.

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Formulation of Antipathogenic Compositions

The present invention further embraces the preparation of antifungal compositions in which the active ingredient is the antibiotic substance produced by the recombinant biocontrol agent of the present invention or alternatively a suspension or concentrate of the microorganism. The active ingredient is homogeneously mixed with one or more compounds or groups of compounds described herein. The present invention also relates to methods of protecting plants against a phytopathogen, which comprise application of the active ingredient, or antifungal compositions containing the active ingredient, to plants in amounts which inhibit said phytopathogen.

The active ingredients of the present invention are normally applied in the form of compositions and can be applied to the crop area or plant to be treated, simultaneously or in succession, with further compounds. These compounds can be both fertilizers or micronutrient donors or other preparations that influence plant growth. They can also be selective herbicides, insecticides, fungicides, bactericides, nematocides, molluscicides or mixtures of several of these preparations, if desired together with further carriers, surfactants or application-promoting adjuvants customarily employed in the art of formulation. Suitable carriers and adjuvants can be solid or liquid and correspond to the substances ordinarily employed in formulation technology, e.g. natural or regenerated mineral substances, solvents, dispersants, wetting agents, tackifiers, binders or fertilizers.

A preferred method of applying active ingredients of the present invention or an agrochemical composition which contains at least one of the active ingredients is leaf application. The number of applications and the rate of application depend on the intensity of infestation by the corresponding phytopathogen (type of fungus). However, the active ingredients can also penetrate the plant through the roots via the soil (systemic action) by impregnating the locus of the plant with a liquid composition, or by applying the compounds in solid form to the soil, e.g. in granular form (soil application). The active ingredients may also be applied to seeds (coating) by impregnating the seeds either with a liquid formulation containing active ingredients, or coating them with a solid formulation. In special cases, further types of application are also possible, for example, selective treatment of the plant stems or buds.

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The active ingredients are used in unmodified form or, preferably, together with the adjuvants conventionally employed in the art of formulation, and are therefore formulated in known manner to emulsifiable concentrates, coatable pastes, directly sprayable or dilutable solutions, dilute emulsions, wettable powders, soluble powders, dusts, granulates, and also encapsulations, for example, in polymer substances. Like the nature of the compositions, the methods of application, such as spraying, atomizing, dusting, scattering or pouring, are chosen in accordance with the intended objectives and the prevailing circumstances. Advantageous rates of application are normally from 50 g to 5 kg of active ingredient (a.i.) per hectare, preferably from 100 g to 2 kg a.i./ha, most preferably from 200 g to 500 g a.i./ha.

The formulations, compositions or preparations containing the active ingredients and, where appropriate, a solid or liquid adjuvant, are prepared in known manner, for example by homogeneously mixing and/or grinding the active ingredients with extenders, for example solvents, solid carriers and, where appropriate, surface-active compounds (surfactants).

Suitable solvents include aromatic hydrocarbons, preferably the fractions having 8 to 12 carbon atoms, for example, xylene mixtures or substituted naphthalenes, phthalates such as dibutyl phthalate or dioctyl phthalate, aliphatic hydrocarbons such as cyclohexane or paraffins, alcohols and glycols and their ethers and esters, such as ethanol, ethylene glycol monomethyl or monoethyl ether, ketones such as cyclohexanone, strongly polar solvents such as N-methyl-2-pyrrolidone, dimethyl sulfoxide or dimethyl formamide, as well as epoxidized vegetable oils such as epoxidized coconut oil or soybean oil; or water.

The solid carriers used e.g. for dusts and dispersible powders, are normally natural mineral fillers such as calcite, talcum, kaolin, montmorillonite or attapulgite. In order to improve the physical properties it is also possible to add highly dispersed silicic acid or highly dispersed absorbent polymers. Suitable granulated adsorptive carriers are porous types, for example pumice, broken brick, sepiolite or bentonite; and suitable nonsorbent carriers are materials such as calcite or sand. In addition, a great number of pregranulated materials of inorganic or organic nature can be used, e.g. especially dolomite or pulverized plant residues.

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Depending on the nature of the active ingredient to be used in the formulation, suitable surface-active compounds are nonionic, cationic and/or anionic surfactants having good emulsifying, dispersing and wetting properties. The term "surfactants" will also be understood as comprising mixtures of surfactants.

Suitable anionic surfactants can be both water-soluble soaps and water-soluble synthetic surface-active compounds.

Suitable soaps are the alkali metal salts, alkaline earth metal salts or unsubstituted or substituted ammonium salts of higher fatty acids (chains of 10 to 22 carbon atoms), for example the sodium or potassium salts of oleic or stearic acid, or of natural fatty acid mixtures which can be obtained for example from coconut oil or tallow oil. The fatty acid methyltaurin salts may also be used.

More frequently, however, so-called synthetic surfactants are used, especially fatty sulfonates, fatty sulfates, sulfonated benzimidazole derivatives or alkylarylsulfonates.

The fatty sulfonates or sulfates are usually in the form of alkali metal salts, alkaline earth metal salts or unsubstituted or substituted ammoniums salts and have a 8 to 22 carbon alkyl radical which also includes the alkyl moiety of alkyl radicals, for example, the sodium or calcium salt of lignonsulfonic acid, of dodecylsulfate or of a mixture of fatty alcohol sulfates obtained from natural fatty acids. These compounds also comprise the salts of sulfuric acid esters and sulfonic acids of fatty alcohol/ethylene oxide adducts. The sulfonated benzimidazole derivatives preferably contain 2 sulfonic acid groups and one fatty acid radical containing 8 to 22 carbon atoms. Examples of alkylarylsulfonates are the sodium, calcium or triethanolamine salts of dodecylbenzenesulfonic acid, dibutyl-naphthalenesulfonic acid, or of a naphthalenesulfonic acid/formaldehyde condensation product. Also suitable are corresponding phosphates, e.g. salts of the phosphoric acid ester of an adduct of p-nonylphenol with 4 to 14 moles of ethylene oxide.

Non-ionic surfactants are preferably polyglycol ether derivatives of aliphatic or cycloaliphatic alcohols, or saturated or unsaturated fatty acids and alkylphenols, said derivatives

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containing 3 to 30 glycol ether groups and 8 to 20 carbon atoms in the (aliphatic) hydrocarbon moiety and 6 to 18 carbon atoms in the alkyl moiety of the alkylphenols.

Further suitable non-ionic surfactants are the water-soluble adducts of polyethylene oxide with polypropylene glycol, ethylenediamine propylene glycol and alkylpolypropylene glycol containing 1 to 10 carbon atoms in the alkyl chain, which adducts contain 20 to 250 ethylene glycol ether groups and 10 to 100 propylene glycol ether groups. These compounds usually contain 1 to 5 ethylene glycol units per propylene glycol unit.

Representative examples of non-ionic surfactants are nonylphenolpolyethoxyethanols, castor oil polyglycol ethers, polypropylene/polyethylene oxide adducts, tributylphenoxypolyethoxyethanol, polyethylene glycol and octylphenoxyethoxyethanol. Fatty acid esters of polyoxyethylene sorbitan and polyoxyethylene sorbitan trioleate are also suitable non-ionic surfactants.

Cationic surfactants are preferably quaternary ammonium salts which have, as N-substituent, at least one C8-C22 alkyl radical and, as further substituents, lower unsubstituted or halogenated alkyl, benzyl or lower hydroxyalkyl radicals. The salts are preferably in the form of halides, methylsulfates or ethylsulfates, e.g. stearyltrimethylammonium chloride or benzyldi(2-chloroethyl)ethylammonium bromide.

The surfactants customarily employed in the art of formulation are described, for example, in "McCutcheon's Detergents and Emulsifiers Annual," MC Publishing Corp. Ringwood, New Jersey, 1979, and Sisely and Wood, "Encyclopedia of Surface Active Agents," Chemical Publishing Co., Inc. New York, 1980.

The agrochemical compositions usually contain from about 0.1 to about 99 %, preferably about 0.1 to about 95 %, and most preferably from about 3 to about 90 % of the active ingredient, from about 1 to about 99.9 %, preferably from about 1 to about 99 %, and most preferably from about 5 to about 95 % of a solid or liquid adjuvant, and from about 0 to about 25 %, preferably about 0.1 to about 25 %, and most preferably from about 0.1 to about 20 % of a surfactant.

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Whereas commercial products are preferably formulated as concentrates, the end user will normally employ dilute formulations.

EXAMPLES

The following examples serve as further description of the invention and methods for practicing the invention. They are not intended as being limiting, rather as providing guidelines on how the invention may be practiced.

A. Identification of Microorganisms which Produce Antipathogenic Substances

Microorganisms can be isolated from many sources and screened for their ability to inhibit fungal or bacterial growth *in vitro*. Typically the microorganisms are diluted and plated on medium onto or into which fungal spores or mycelial fragments, or bacteria have been or are to be introduced. Thus, zones of clearing around a newly isolated bacterial colony are indicative of antipathogenic activity.

Example 1: Isolation of Microorganisms with Anti-*Rhizoctonia* Properties from Soil

A gram of soil (containing approximately 10^6 - 10^8 bacteria) is suspended in 10 ml sterile water. After vigorously mixing, the soil particles are allowed to settle. Appropriate dilutions are made and aliquots are plated on nutrient agar plates (or other growth medium as appropriate) to obtain 50-100 colonies per plate. Freshly cultured *Rhizoctonia* mycelia are fragmented by blending and suspensions of fungal fragments are sprayed on to the agar plates after the bacterial colonies have grown to be just visible. Bacterial isolates with antifungal activities can be identified by the fungus-free zones surrounding them upon further incubation of the plates.

The production of bioactive metabolites by such isolates is confirmed by the use of culture filtrates in place of live colonies in the plate assay described above. Such bioassays can also be used for monitoring the purification of the metabolites. Purification may start with an organic solvent extraction step and depending on whether the active principle is extracted into the organic phase or left in the aqueous phase, different chromatographic steps follow.

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These chromatographic steps are well known in the art. Ultimately, purity and chemical identity are determined using spectroscopic methods.

B. Cloning Antipathogenic Biosynthetic Genes from Microorganisms

Example 2: Shotgun Cloning Antipathogenic Biosynthetic Genes from their Native Source

Related biosynthetic genes are typically located in close proximity to each other in microorganisms and more than one open reading frame is often encoded by a single operon. Consequently, one approach to the cloning of genes which encode enzymes in a single biosynthetic pathway is the transfer of genome fragments from a microorganism containing said pathway to one which does not, with subsequent screening for a phenotype conferred by the pathway.

In the case of biosynthetic genes encoding enzymes leading to the production of an antipathogenic substance (APS), genomic DNA of the antipathogenic substance producing microorganism is isolated, digested with a restriction endonuclease such as *Sau3A*, size fractionated for the isolation of fragments of a selected size (the selected size depends on the vector being used), and fragments of the selected size are cloned into a vector (e.g. the *BamHI* site of a cosmid vector) for transfer to *E. coli*. The resulting *E. coli* clones are then screened for those which are producing the antipathogenic substance. Such screens may be based on the direct detection of the antipathogenic substance, such as a biochemical assay.

Alternatively, such screens may be based on the adverse effect associated with the antipathogenic substance upon a target pathogen. In these screens, the clones producing the antipathogenic substance are selected for their ability to kill or retard the growth of the target pathogen. Such an inhibitory activity forms the basis for standard screening assays well known in the art, such as screening for the ability to produce zones of clearing on a bacterial plate impregnated with the target pathogen (eg. spores where the target pathogen is a fungus, cells where the target pathogen is a bacterium). Clones selected for their antipathogenic activity can then be further analyzed to confirm the presence of the

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antipathogenic substance using the standard chemical and biochemical techniques appropriate for the particular antipathogenic substance.

Further characterization and identification of the genes encoding the biosynthetic enzymes for the antipathogenic substance is achieved as follows. DNA inserts from positively identified *E. coli* clones are isolated and further digested into smaller fragments. The smaller fragments are then recloned into vectors and reinserted into *E. coli* with subsequent reassaying for the antipathogenic phenotype. Alternatively, positively identified clones can be subjected to λ ::Tn5 transposon mutagenesis using techniques well known in the art (e.g. de Bruijn & Lupski, *Gene* **27**: 131-149 (1984)). Using this method a number of disruptive transposon insertions are introduced into the DNA shown to confer APS production to enable a delineation of the precise region/s of the DNA which are responsible for APS production. Subsequently, determination of the sequence of the smallest insert found to confer antipathogenic substance production on *E. coli* will reveal the open reading frames required for APS production. These open reading frames can ultimately be disrupted (see below) to confirm their role in the biosynthesis of the antipathogenic substance.

Various host organisms such as *Bacillus* and yeast may be substituted for *E. coli* in the techniques described using suitable cloning vectors known in the art for such host. The choice of host organism has only one limitation; it should not be sensitive to the antipathogenic substance for which the biosynthetic genes are being cloned.

Example 3: Cloning Biosynthetic Genes for an Antipathogenic Substance using Transposon Mutagenesis

In many microorganisms which are known to produce antipathogenic substances, transposon mutagenesis is a routine technique used for the generation of insertion mutants. This technique has been used successfully in *Pseudomonas* (e.g. Lam *et al.*, *Plasmid* **13**:200-204 (1985)), *Bacillus* (e.g. Youngman *et al.*, *Proc. Natl. Acad. Sci. USA* **80**:2305-2309 (1983)), *Staphylococcus* (e.g. Pattee, *J. Bacteriol.* **145**:479-488 (1981)), and *Streptomyces* (e.g. Schauer *et al.*, *J. Bacteriol.* **173**:5060-5067 (1991)), among others. The main requirement for the technique is the ability to introduce a transposon containing plasmid into the microorganism enabling the transposon to insert itself at a random position in the genome. A large library of insertion mutants is created by introducing a transposon

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carrying plasmid into a large number of microorganisms. Introduction of the plasmid into the microorganism can be by any appropriate standard technique such as conjugation, direct gene transfer techniques such as electroporation.

Once a transposon library has been created in the manner described above, the transposon insertion mutants are assayed for production of the APS. Mutants which do not produce the APS would be expected to predominantly occur as the result of transposon insertion into gene sequences required for APS biosynthesis. These mutants are therefore selected for further analysis.

DNA from the selected mutants which is adjacent to the transposon insert is then cloned using standard techniques. For instance, the host DNA adjacent to the transposon insert may be cloned as part of a library of DNA made from the genomic DNA of the selected mutant. This adjacent host DNA is then identified from the library using the transposon as a DNA probe. Alternatively, if the transposon used contains a suitable gene for antibiotic resistance, then the insertion mutant DNA can be digested with a restriction endonuclease which will be predicted not to cleave within this gene sequence or between its sequence and the host insertion point, followed by cloning of the fragments thus generated into a microorganism such as *E. coli* which can then be subjected to selection using the chosen antibiotic.

Sequencing of the DNA beyond the inserted transposon reveals the adjacent host sequences. The adjacent sequences can in turn be used as a hybridization probe to reclone the undisrupted native host DNA using a non-mutant host library. The DNA thus isolated from the non-mutant is characterized and used to complement the APS deficient phenotype of the mutant. DNA which complements may contain either APS biosynthetic genes or genes which regulate all or part of the APS biosynthetic pathway. To be sure isolated sequences encode biosynthetic genes they can be transferred to a heterologous host which does not produce the APS and which is insensitive to the APS (such as *E. coli*). By transferring smaller and smaller pieces of the isolated DNA and the sequencing of the smallest effective piece, the APS genes can be identified. Alternatively, positively identified clones can be subjected to λ ::Tn5 transposon mutagenesis using techniques well known in the art (e.g. de Bruijn & Lupski, *Gene* 27: 131-149 (1984)). Using this method a number of

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disruptive transposon insertions are introduced into the DNA shown to confer APS production to enable a delineation of the precise region/s of the DNA which are responsible for APS production. These latter steps are undertaken in a manner analagous to that described in example 1. In order to avoid the possibility of the cloned genes not being expressed in the heterologous host due to the non-functioning of their heterologous promoter, the cloned genes can be transferred to an expression vector where they will be fused to a promoter known to function in the heterologous host. In the case of *E. coli* an example of a suitable expression vector is pKK223 which utilizes the *tac* promoter. Similar suitable expression vectors also exist for other hosts such as yeast and are well known in the art. In general such fusions will be easy to undertake because of the operon-type organization of related genes in microorganisms and the likelihood that the biosynthetic enzymes required for APS biosynthesis will be encoded on a single transcript requiring only a single promoter fusion.

Example 4: Cloning Antipathogenic Biosynthetic Genes using Mutagenesis and Complementation

A similar method to that described above involves the use of non-insertion mutagenesis techniques (such as chemical mutagenesis and radiation mutagenesis) together with complementation. The APS producing microorganism is subjected to non-insertion mutagenesis and mutants which lose the ability to produce the APS are selected for further analysis. A gene library is prepared from the parent APS-producing strain. One suitable approach would be the ligation of fragments of 20-30 kb into a vector such as pVK100 (Knauf *et al.* Plasmid 8: 45-54 (1982)) into *E. coli* harboring the *tra+* plasmid pRK2013 which would enable the transfer by triparental conjugation back to the selected APS-minus mutant (Ditta *et al.* Proc. Natl. Acad. Sci. USA 77: 7247-7351 (1980)). A further suitable approach would be the transfer back to the mutant of the genes library via electroporation. In each case subsequent selection is for APS production. Selected colonies are further characterized by the retransformation of APS-minus mutant with smaller fragments of the complementing DNA to identify the smallest successfully complementing fragment which is then subjected to sequence analysis. As with example 2, genes isolated by this procedure may be biosynthetic genes or genes which regulate the entire or part of the APS biosynthetic pathway. To be sure that the isolated sequences encode biosynthetic genes they can be transferred to a heterologous host which does not produce the APS and is

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insensitive to the APS (such as *E. coli*). These latter steps are undertaken in a manner analogous to that described in example 2.

Example 5: Cloning Antipathogenic Biosynthetic Genes by Exploiting Regulators which Control the Expression of the Biosynthetic Genes

A further approach in the cloning of APS biosynthetic genes relies on the use of regulators which control the expression of these biosynthetic genes. A library of transposon insertion mutants is created in a strain of microorganism which lacks the regulator or has had the regulator gene disabled by conventional gene disruption techniques. The insertion transposon used carries a promoter-less reporter gene (*e.g. lacZ*). Once the insertion library has been made, a functional copy of the regulator gene is transferred to the library of cells (*e.g.* by conjugation or electroporation) and the plated cells are selected for expression of the reporter gene. Cells are assayed before and after transfer of the regulator gene. Colonies which express the reporter gene only in the presence of the regulator gene are insertions adjacent to the promoter of genes regulated by the regulator. Assuming the regulator is specific in its regulation for APS-biosynthetic genes, then the genes tagged by this procedure will be APS-biosynthetic genes. These genes can then be cloned and further characterized using the techniques described in example 2.

Example 6: Cloning Antipathogenic Biosynthetic Genes by Homology

Standard DNA techniques can be used for the cloning of novel antipathogenic biosynthetic genes by virtue of their homology to known genes. A DNA library of the microorganism of interest is made and then probed with radiolabelled DNA derived from the gene/s for APS biosynthesis from a different organism. The newly isolated genes are characterized and sequenced and introduced into a heterologous microorganism or a mutant APS-minus strain of the native microorganisms to demonstrate their conferral of APS production.

C. Cloning of Pyrrolnitrin Biosynthetic Genes from *Pseudomonas*

Pyrrolnitrin is a phenylpyrole compound produced by various strains of *Pseudomonas fluorescens*. *P. fluorescens* strains which produce pyrrolnitrin are effective biocontrol strains against *Rhizoctonia* and *Pythium* fungal pathogens (WO 94/01561). The biosynthesis of pyrrolnitrin is postulated to start from tryptophan (Chang *et al.* J. Antibiotics 34: 555-566 (1981)).

Example 7: Use of the *gafA* Regulator Gene for the Isolation of Pyrrolnitrin Biosynthetic Genes from *Pseudomonas*

The gene cluster encoding pyrrolnitrin biosynthetic enzymes was isolated using the basic principle described in example 5 above. The regulator gene used in this isolation procedure was the *gafA* gene from *Pseudomonas fluorescens* and is known to be part of a two-component regulatory system controlling certain biocontrol genes in *Pseudomonas*. The *gafA* gene is described in detail in WO 94/01561 which is hereby incorporated by reference in its entirety. *gafA* is further described in Gaffney *et al.* (Molecular Plant-Microbe Interactions 7: 455-463, 1994, also hereby incorporated in its entirety by reference) where it is referred to as "ORF5". The *gafA* gene has been shown to regulate pyrrolnitrin biosynthesis, chitinase, gelatinase and cyanide production. Strains which lack the *gafA* gene or which express the gene at low levels (and in consequence *gafA*-regulated genes also at low levels) are suitable for use in this isolation technique.

Example 8: Isolation of Pyrrolnitrin Biosynthesis Genes in *Pseudomonas*

The transfer of the *gafA* gene from MOCG 134 to closely related non-pyrrolnitrin producing wild-type strains of *Pseudomonas fluorescens* results in the ability of these strains to produce pyrrolnitrin. (Gaffney *et al.*, MPMI (1994)); see also Hill *et al.* Applied And Environmental Microbiology 60 78-85 (1994)). This indicates that these closely related strains have the structural genes needed for pyrrolnitrin biosynthesis but are unable to produce the compound without activation from the *gafA* gene. One such closely related strain, MOCG133, was used for the identification of the pyrrolnitrin biosynthesis genes. The transposon TnCIB116 (Lam, New Directions in Biological Control: Alternatives for Suppressing Agricultural Pests and Diseases, pp 767-778, Alan R. Liss, Inc. (1990)) was used to mutagenize MOCG133. This transposon, a Tn5 derivative, encodes kanamycin resistance and contains a promoterless lacZ reporter gene near one end. The transposon was introduced into MOCG133 by conjugation, using the plasmid vector pCIB116 (Lam, New Directions in Biological Control: Alternatives for Suppressing Agricultural Pests and Diseases, pp 767-778, Alan R. Liss, Inc. (1990)) which can be mobilized into MOCG133, but cannot replicate in that organism. Most, if not all, of the kanamycin resistant transconjugants were therefore the result of transposition of TnCIB116 into different sites in the MOCG133 genome. When the transposon integrates into the bacterial chromosome behind an active promoter the lacZ reporter gene is activated. Such gene activation can be

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monitored visually by using the substrate X-gal, which releases an insoluble blue product upon cleavage by the *lacZ* gene product. Kanamycin resistant transconjugants were collected and arrayed on master plates which were then replica plated onto lawns of *E. coli* strain S17-1 (Simon *et al.*, Bio/technology 1:784-791 (1983)) transformed with a plasmid carrying the wide host range RK2 origin of replication, a gene for tetracycline selection and the *gafA* gene. *E. coli* strain S17-1 contains chromosomally integrated *tra* genes for conjugal transfer of plasmids. Thus, replica plating of insertion transposon mutants onto a lawn of the S17-1/*gafA E. coli* results in the transfer to the insertion transposon mutants of the *gafA*-carrying plasmid and enables the activity of the *lacZ* gene to be assayed in the presence of the *gafA* regulator (expression of the host *gafA* is insufficient to cause *lacZ* expression, and introduction of *gafA* on a multicopy plasmid is more effective). Insertion mutants which had a "blue" phenotype (i.e. *lacZ* activity) only in the presence of *gafA* were identified. In these mutants, the transposon had integrated within genes whose expression were regulated by *gafA*. These mutants (with introduced *gafA*) were assayed for their ability to produce cyanide, chitinase, and pyrrolnitrin (as described in Gaffney *et al.*, 1994 MPMI, in press) --activities known to be regulated by *gafA* (Gaffney *et al.*, 1994 MPMI, in press). One mutant did not produce pyrrolnitrin but did produce cyanide and chitinase, indicating that the transposon had inserted in a genetic region involved only in pyrrolnitrin biosynthesis. DNA sequences flanking one end of the transposon were cloned by digesting chromosomal DNA isolated from the selected insertion mutant with *XhoI*, ligating the fragments derived from this digestion into the *XhoI* site of pSP72 (Promega, cat. # P2191) and selecting the *E. coli* transformed with the products of this ligation on kanamycin. The unique *XhoI* site within the transposon cleaves beyond the gene for kanamycin resistance and enabled the flanking region derived from the parent MOCG 133 strain to be concurrently isolated on the same *XhoI* fragment. In fact the *XhoI* site of the flanking sequence was found to be located approximately 1 kb away from the end on the transposon. A subfragment of the cloned *XhoI* fragment derived exclusively from the ~1 kb flanking sequence was then used to isolate the native (i.e. non-disrupted) gene region from a cosmid library of strain MOCG 134. The cosmid library was made from partially *Sau3A* digested MOCG 134 DNA, size selected for fragments of between 30 and 40 kb and cloned into the unique *BamHI* site of the cosmid vector pCIB119 which is a derivative of c2XB (Bates & Swift, Gene 26: 137-146 (1983)) and pRK290 (Ditta *et al.* Proc. Natl. Acad. Sci. USA 77: 7247-7351 (1980)). pCIB119 is a double-*cos* site cosmid vector which has the

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wide host range RK2 origin of replication and can therefore replicate in *Pseudomonas* as well as *E. coli*. Several clones were isolated from the MOCG 134 cosmid clone library using the ~1 kb flanking sequence as a hybridization probe. Of these one clone was found to restore pyrrolnitrin production to the transposon insertion mutant which had lost its ability to produce pyrrolnitrin. This clone had an insertion of ~32 kb and was designated pCIB169. A viable culture of *E. coli* DH5 α comprising cosmid clone pCIB169 has been deposited with the Agricultural Research Culture Collection (NRRL) at 1815 N. University Street, Peoria, Illinois 61604 U.S.A. on May 20, 1994, under the accession number NRRL B-21256.

Example 9: Mapping and Tn5 Mutagenesis of pCIB169

The 32 kb insert of clone pCIB169 was subcloned into pCIB189 in *E. coli* HB101, a derivative of pBR322 which contains a unique *NotI* cloning site. A convenient *NotI* site within the 32 kb insert as well as the presence of *NotI* sites flanking the *BamHI* cloning site of the parent cosmid vector pCIB119 allowed the subcloning of fragments of 14 and 18 kb into pCIB189. These clones were both mapped by restriction digestion and figure 1 shows the result of this. λ Tn5 transposon mutagenesis was carried out on both the 14 and 18 kb subclones using techniques well known in the art (*e.g.* de Bruijn & Lupski, *Gene* **27**: 131-149 (1984)). λ Tn5 phage conferring kanamycin resistance was used to transfect both the 14 and the 18 kb subclones described above. λ Tn5 transfections were done at a multiplicity of infection of 0.1 with subsequent selection on kanamycin. Following mutagenesis plasmid DNA was prepared and retransformed into *E. coli* HB101 with kanamycin selection to enable the isolation of plasmid clones carrying Tn5 insertions. A total of 30 independent Tn5 insertions were mapped along the length of the 32 kb insert (see figure 2). Each of these insertions was crossed into MOCG 134 via double homologous recombination and verified by Southern hybridization using the Tn5 sequence and the pCIB189 vector as hybridization probes to demonstrate the occurrence of double homologous recombination *i.e.* the replacement of the wild-type MOCG 134 gene with the Tn5-insertion gene. Pyrrolnitrin assays were performed on each of the insertions that were crossed into MOCG 134 and a genetic region of approximately 6 kb was identified to be involved in pyrrolnitrin production (see figures 3 and 5). This region was found to be centrally located in pCIB169 and was easily subcloned as an *XbaI/NotI* fragment into pBluescript II KS (Promega). The *XbaI/NotI* subclone was designated pPRN5.9X/N (see figure 4).

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Example 10: Identification of Open Reading Frames in the Cloned Genetic Region

The genetic region involved in pyrrolnitrin production was subcloned into six fragments for sequencing in the vector pBluescript II KS (see figure 4). These fragments spanned the ~6 kb *Xba*I/*Not*I fragment described above and extended from the *Eco*RI site on the left side of figure 4 to the rightmost *Hind*III site (see figure 4). The sequence of the inserts of clones pPRN1.77E, pPRN1.01E, pPRN1.24E, pPRN2.18E, pPRN0.8H/N, and pPRN2.7H was determined using the Taq DyeDeoxy Terminator Cycle Sequencing Kit supplied by Applied Biosystems, Inc., Foster City, CA. following the protocol supplied by the manufacturer. Sequencing reactions were run on an Applied Biosystems 373A Automated DNA Sequencer and the raw DNA sequence was assembled and edited using the "INHERIT" software package also from Applied Biosystems, Inc.. A contiguous DNA sequence of 9.7 kb was obtained corresponding to the *Eco*RI/*Hind*III fragment of Figure 3 and bounded by *Eco*RI site # 2 and *Hind*III site # 2 depicted in figure 4.

DNA sequence analysis was performed on the contiguous 9.7 kb sequence using the GCG software package from Genetics Computer Group, Inc. Madison, WI. The pattern recognition program "FRAMES" was used to search for open reading frames (ORFs) in all six translation frames of the DNA sequence. Four open reading frames were identified using this program and the codon frequency table from ORF2 of the *gafA* gene region which was previously published (*WO 94/05793*; figure 5). These ORFs lie entirely within the ~6 kb *Xba*I/*Not*I fragment referred to in example 9 (figure 4) and are contained within the sequence disclosed as SEQ ID NO:1. By comparing the codon frequency usage table from MOCG134 DNA sequence of the *gafA* region to these four open reading frames, very few rare codons were used indicating that codon usage was similar in both of these gene regions. This strongly suggested that the four open reading frames were real. At a 3' position to the fourth reading frame numerous p-independent stem loop structures were found suggesting a region where transcription could be stopped. It was thus apparent that all four ORFs were translated from a single transcript. Sequence data obtained for the regions beyond the four identified ORFs revealed a fifth open reading frame which was subsequently determined to not be involved in pyrrolnitrin synthesis based on *E. coli* expression studies.

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For each open reading frame (ORF) in the pyrrolnitrin gene cluster multiple putative translation start sites were identified by the presence of an in-frame start codon (ATG or GTG) and an upstream ribosome binding site. A complementation approach was used to identify the actual translation start site for each gene. PCR primers were synthesized to amplify segments of each *pm* gene from upstream of one of the putative ribosome binding sites to downstream of the stop codon (Table 1). The plasmid pPRN18Not (1506 CIP3, Figure 4) was used as the template for PCR reactions. The PCR products were cloned in the vector pRK(KK223-3MCS) which consists of the *P*_{tac} promoter and *rrs* terminator from pKK223-3 (Pharmacia) and pRK290 backbone. Plasmids containing each construct were mobilized into the respective ORF-deletion mutants of MOCG134 as described in example 12 and by triparental matings using the helper plasmid pRK290 in *E. coli* HB101. Transconjugants were selected by plating on *Pseudomonas* minimal medium supplemented with 30 mg/l tetracycline. The presence of the plasmids and correct orientations of the inserted PCR product were verified by plasmid DNA preparation, restriction digestion and agarose gel electrophoresis. Pyrrolnitrin production was determined by extraction and TLC assay as in example 11. For each *pm* gene the shortest clone restoring pyrrolnitrin production (i.e., complementing the ORF deletion) was judged to contain the actual translation initiation site. Thus, the initiation codons were identified as follows: ORF1 - ATG at nucleotide position 423, ORF2 - GTG at nucleotide position 2026, ORF3 - ATG at nucleotide position 3166, and ORF4 - ATG at nucleotide position 4894. The pattern "FRAMES" computer program used to indentify the open reading frames only recognizes ATG start codons. Using the complementation approach describe here it was determined that ORF2 actually starts with a GTG codon at nucleotide position 2039 and is thus longer than the open reading frame identified by the "FRAMES" program.

Table 1: DNA constructs and hosts used to identify translation initiation sites in the pyrrolnitrin gene cluster^a.

Construct	Start of amplified segment	Putative start codon ^b	Stop codon ^c	End of amplified segment	Host strain ^d	Pyrrolnitrin production
ORF1-1	294	357	2039	2056	ORF1D	+
ORF1-2	396	423	2039	2056	ORF1D	+
ORF1-3	438	477	2039	2056	ORF1D	-
ORF2-1	2026	2039	3076	3166	ORF2D	+
ORF2-2	2145	2162	3076	3166	ORF2D	-
ORF2-3	2249	2215	3076	3166	ORF2D	-
ORF3-1	3130	3166	4869	4904	ORF3D	+
ORF3-2	3207	3235	4869	4904	ORF3D	-
ORF3-3	3329	3355	4869	4904	ORF3D	-
ORF4-1	4851	4894	5985	6122	ORF4D	+
ORF4-2	4967	4990	5985	6122	ORF4D	-
ORF4-3	5014	5086	5985	6122	ORF4D	-

^a All nucleotide position numbers refer to the Sequence of the Pyrrolnitrin Gene Cluster given in SEQ ID No. 1

^b The first base of the putative start codon

^c The last base of the stop codon

^d ORF deletion mutants are described in Example 12

Example 11: Expression of Pyrrolnitrin Biosynthetic Genes in *E. coli*

To determine if only four genes were needed for pyrrolnitrin production, these genes were transferred into *E. coli* which was then assayed for pyrrolnitrin production. The expression vector pKK223-3 was used to over-express the cloned operon in *E. coli*. (Brosius & Holy, Proc. Natl. Acad. Sci. USA 81: 6929 (1984)). pKK223-3 contains a strong *tac* promoter which, in the appropriate host, is regulated by the *lac* repressor and induced by the addition of isopropyl- β -D-thiogalactoside (IPTG) to the bacterial growth medium. This vector was modified by the addition of further useful restriction sites to the existing multiple cloning site to facilitate the cloning of the ~6 kb *Xba*I/*Not*I fragment (see example 7 and figure 4) and a

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10 kb *Xba*I/*Kpn*I fragment (see figure 4) for expression studies. In each case the cloned fragment was under the control of the *E. coli tac* promoter (with IPTG induction), but was cloned in a transcriptional fusion so that the ribosome binding site used would be that derived from *Pseudomonas*. Each of these clones was transformed into *E. coli* XL1-blue host cells and induced with 2.5 mM IPTG before being assayed for pyrrolnitrin by thin layer chromatography. Cultures were grown for 24 h after IPTG induction in 10 ml L broth at 37 C with rapid shaking, then extracted with an equal volume of ethyl acetate. The organic phase was recovered, allowed to evaporated under vacuum and the residue dissolved in 20 μ l of methanol. Silica gel thin layer chromatography (TLC) plates were spotted with 10 μ l of extract and run with toluene as the mobile phase. The plates were allowed to dry and sprayed with van Urk's reagent to visualize. Urk's reagent comprises 1g p-Dimethylaminobenzaldehyde in 50 ml 36% HCL and 50 ml 95% ethanol. Under these conditions pyrrolnitrin appears as a purple spot on the TLC plate. This assay confirmed the presence of pyrrolnitrin in both of the expression constructs. HPLC and mass spectrometry analysis further confirmed the presence of pyrrolnitrin in both of the extracts. HPLC analysis can be undertaken directly after redissolving in methanol (in this case the sample is redissolved in 55 % methanol) using a Hewlett Packard Hypersil ODS column (5 μ M) of dimensions 100 x 2.1 mm.. Pyrrolnitrin elutes after about 14 min.

Example 11a: Construction of strain MOCG134cPm having pyrrolnitrin biosynthetic genes under a constitutive promoter

Transcription of the pyrrolnitrin biosynthetic genes is regulated by *gafA*. Thus, transcription and Pyrrolnitrin production does not reach high levels until late log and stationary growth phase. To increase pyrrolnitrin biosynthesis in earlier growth phases the endogenous promoter was replaced with the strong constitutive *E. coli tac* promoter. The Pm genes were cloned between the *tac* promoter and a strong terminator sequence as described in example 11 above. The resulting synthetic operon was inserted into a genomic clone that had the Pm biosynthetic genes deleted but has homologous sequences both upstream and downstream of the insertion site. This clone was mobilized into strain MOCG134_Pm, a deletion mutant of the genes Pm A-D. The Pm genes under the control of the constitutive *tac* promoter were inserted into the bacterial chromosome via double homologous recombination. The resultant strain MOCG134cPm was shown to produce Pyrrolnitrin earlier than the wild-type strain.

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Pyrrolnitrin production of the wild type strain MOCG134, of strain MOCG134cPm, and of a strain containing plasmid borne PRN genes under the control of the *tac* promoter (MOCG134pPm) was assayed at various time points (14, 17, 20, 23 and 26 hours growth). Cultures were inoculated with a 1/10,000 dilution of a stationary phase culture, Pyrrolnitrin was extracted with ethyl acetate, and the amount of Pyrrolnitrin was determined by integrating the peak area of Pyrrolnitrin detected by HPLC at 212 nm. The results shown in Table 3 clearly indicate that strains containing the Pm genes under the control of the *tac* promoter produce Pyrrolnitrin much earlier than the wild type MOCG134 strain. The new strains produce Pyrrolnitrin independent of *gafA* and are useful as new biocontrol strains.

Table 3: Pyrrolnitrin production of different strains at different time points

time of growth (hours)	amount Pyrrolnitrin produced (peak area)		
	MOCG134	MOCG134cPm	MOCG134pPm
14	1250	7100	18300
17	3500	14600	26700
20	9600	16600	32100
23	17500	18900	31000
26	25000	22500	33500

Example 12: Construction of Pyrrolnitrin Gene Deletion Mutants

To further demonstrate the involvement of the 4 ORFs in pyrrolnitrin biosynthesis, independent deletions were created in each ORF and transferred back into *Pseudomonas fluorescens* strain MOCG134 by homologous recombination. The plasmids used to generate deletions are depicted in Figure 4 and the positions of the deletions are shown in Figure 6. Each ORF is identified within the sequence disclosed as SEQ ID NO:1.

ORF1 (SEQ ID NO:2):

The plasmid pPRN1.77E was digested with *Mlu*I to liberate a 78 bp fragment internally from ORF1. The remaining 4.66 kb vector-containing fragment was recovered, religated with T4 DNA ligase, and transformed into the *E. coli* host strain DH5 α . This new plasmid was linearized with *Mlu*I and the Klenow large fragment of DNA polymerase I was used to create blunt ends (Maniatis *et al.* Molecular Cloning, Cold Spring Harbor Laboratory

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(1982)). The neomycin phosphotransferase II (NPTII) gene cassette from pUC4K (Pharmacia) was ligated into the plasmid by blunt end ligation and the new construct, designated pBS(ORF1Δ), was transformed into DH5α. The construct contained a 78 bp deletion of ORF1 at which position the NPTII gene conferring kanamycin resistance had been inserted. The insert of this plasmid (*i.e.* ORF1 with NPTII insertion) was then excised from the pBluescript II KS vector with *EcoRI*, ligated into the *EcoRI* site of the vector pBR322 and transformed into the *E. coli* host strain HB101. The new plasmid was verified by restriction enzyme digestion and designated pBR322(ORF1Δ).

ORF2 (SEQ ID NO:3):

The plasmids pPRN1.24E and pPRN1.01E containing contiguous *EcoRI* fragments spanning ORF2 were double digested with *EcoRI* and *XhoI*. The 1.09 kb fragment from pPRN1.24E and the 0.69 Kb fragment from pPRN1.01E were recovered and ligated together into the *EcoRI* site of pBR322. The resulting plasmid was transformed into the host strain DH5α and the construct was verified by restriction enzyme digestion and electrophoresis. The plasmid was then linearized with *XhoI*, the NPTII gene cassette from pUC4K was inserted, and the new construct, designated pBR(ORF2Δ), was transformed into HB101. The construct was verified by restriction digestions and agarose gel electrophoresis and contains NPTII within a 472 bp deletion of the ORF2 gene.

ORF3 (SEQ ID NO:4):

The plasmid pPRN2.56Sph was digested with *PstI* to liberate a 350 bp fragment. The remaining 2.22 kb vector-containing fragment was recovered and the NPTII gene cassette from pUC4K was ligated into the *PstI* site. This intermediate plasmid, designated pUC(ORF3Δ), was transformed into DH5α and verified by restriction digestion and agarose gel electrophoresis. The gene deletion construct was excised from pUC with *SphI* and ligated into the *SphI* site of pBR322. The new plasmid, designated pBR(ORF5Δ), was verified by restriction enzyme digestion and agarose gel electrophoresis. This plasmid contains the NPTII gene within a 350 bp deletion of the ORF3 gene.

ORF4 (SEQ ID NO:5):

The plasmid pPRN2.18E/N was digested with *AatII* to liberate 156 bp fragment. The remaining 2.0 kb vector-containing fragment was recovered, religated, transformed into

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DH5 α , and verified by restriction enzyme digestion and electrophoresis. The new plasmid was linearized with *AatII* and T4 DNA polymerase was used to create blunt ends. The NPTII gene cassette was ligated into the plasmid by blunt-end ligation and the new construct, designated pBS(ORF4 Δ), was transformed into DH5 α . The insert was excised from the pBluescript II KS vector with *EcoRI*, ligated into the *EcoRI* site of the vector pBR322 and transformed into the *E. coli* host strain HB101. The identity of the new plasmid, designated pBR(ORF4 Δ), was verified by restriction enzyme digestion and agarose gel electrophoresis. This plasmid contains the NPTII gene within a 264 bp deletion of the ORF4 gene.

Km^R Control:

To control for possible effects of the kanamycin resistance marker, the NPTII gene cassette from pUC4K was inserted upstream of the pyrrolnitrin gene region. The plasmid pPRN2.5S (a subclone of pPRN7.2E) was linearized with *PstI* and the NPTII cassette was ligated into the *PstI* site. This intermediate plasmid was transformed into DH5 α and verified by restriction digestions and agarose gel electrophoresis. The gene insertion construct was excised from pUC with *SphI* and ligated into the *SphI* site of pBR322. The new plasmid, designated pBR(2.5SphIKm^R), was verified by restriction enzyme digestion and agarose gel electrophoresis. It contains the NPTII region inserted upstream of the pyrrolnitrin gene region.

Each of the gene deletion constructs was mobilized into MOCG134 by triparental mating using the helper plasmid pRK2013 in *E. coli* HB101. Gene replacement mutants were selected by plating on *Pseudomonas* Minimal Medium (PMM) supplemented with 50 μ g/ml kanamycin and counterselected on PMM supplemented with 30 μ g/ml tetracycline. Putative perfect replacement mutants were verified by Southern hybridization by probing *EcoRI* digested DNA with pPRN18Not, pBR322 and an NPTII cassette obtained from pUC4K (Pharmacia 1994 catalog no. 27-4958-01). Verification of perfect hybridization was apparent by lack of hybridization to pBR322, hybridization of pPRN18Not to an appropriately size-shifted *EcoRI* fragment (reflecting deletion and insertion of NPTII), hybridization of the NPTII probe to the shifted band, and the disappearance of a band corresponding a deleted fragment.

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After verification, deletion mutants were tested for production of pyrrolnitrin, 2-hexyl-5-propyl-resorcinol, cyanide, and chitinase production. A deletion in any one of the ORFs abolished pyrrolnitrin production, but did not affect production of the other substances. The presence of the NPTII gene cassette in the Km^R control had no effect on the production of pyrrolnitrin, 2-hexyl-5-propyl-resorcinol, cyanide or chitinase. These experiments demonstrated the requirement of each of the four ORFs for pyrrolnitrin production.

Example 12a: Cloning of the coding regions for expression in plants

The coding regions of ORFs 1,2,3, and 4 were designated pmA, pmB, pmC and pmD, respectively. Primers were designed to PCR amplify the coding regions for each pm gene from the start codon to or beyond the stop codon as shown in Table 2. Additionally, the primers were designed to add restriction sites to the ends of the coding regions and in the case of pmB to change the initiation codon for pmB from GTG to ATG. Plasmid pPRN18Not (Figure 4) was used as template for the PCR reactions. The PCR products were cloned into pPEH14 for functional testing. Plasmid pPEH14 is a modification of pRK(KK223-3) which contains a synthetic ribosome binding site 11 to 14 bases upstream of the start codons of the cloned PCR products. The constructs were mobilized into the respective ORF deletion mutants by triparental matings as described earlier. The presence of each plasmid and the correct orientation of the inserted PCR product were confirmed by plasmid DNA extraction, restriction digestion, and agarose gel electrophoresis. Pyrrolnitrin production of the complemented mutants was confirmed as described in example 11.

After the expression of a functional protein by each coding region was verified (i.e., the ability to restore pyrrolnitrin production to an ORF deletion mutant was demonstrated) the clones were sequenced and compared to the sequence of the pyrrolnitrin gene cluster (1506 CIP3). For pmA, pmB and pmC the sequence of the amplified coding regions were identical to the original gene cluster sequences. For pmD there was a single base change at nucleotide position 5605 from G in the original sequence to A in the amplified coding region. This base change results in a change from glycine to serine in the deduced amino acid sequence, but does not affect function of the gene product according to the complementation tests described above.

Table 2: Coding regions of the pm genes^a

Coding region	Start of amplified segment	Start codon ^b	Stop codon ^c	End of amplified segment
pmA	423	423	2039	2055
pmB	2039	2039	3076	3081
pmC	3166	3166	4869	4075
pmD	4894	4894	5985	5985

^a All nucleotide position numbers refer to Sequence ID No. 1

^b The first base of the start codon.

^c The last base of the codon.

Example 12b: Expression of prn genes in plants

The coding regions for each pm gene, described in example 12a above were subcloned into a plant expression cassette consisting of the CaMV 35S promoter and leader and the CaMV 35S terminator flanked by Xba I restriction sites. Each construct comprising promoter, coding region, and terminator was liberated with Xba I, subcloned into the binary transformation vector pCIB200, and then transformed into *Agrobacterium tumefaciens* host strain A136. Tobacco transformation was carried out as described by Horsch et al., Science 227: 1229-1231, 1985). *Arabidopsis* transformation was carried out as described by Lloyd et al, Science 234:464-466, 1986. Plantlets were selected and regenerated on medium containing 100mg/L kanamycin and 500 mg/L carbenecillin.

Tobacco leaf tissue was harvested from individual plants that were suspected to be transformed. *Arabidopsis* leaf tissue from about 10 independent plants suspected to be transformed was pooled for each gene construct used for transformation. RNA was purified by phenol:chloroform extraction and fractionated by formaldehyde gel electrophoresis before blotting onto nylon membranes. Probes to each coding region were made using the random primed labeling method. Hybridization was carried out in 50% formamide at 42°C as described by Sambrook et al., Molecular Cloning, 2nd ed., Cold Spring Harbor Laboratory, 1989.

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For each *pm* gene, transgenic tobacco plants were identified which produced RNA bands hybridizing strongly to the appropriate *pm* gene probe and showing the size expected for a mRNA transcribed from the relevant *pm* gene. Similar bands were also seen in RNA extracted from the pooled samples of *Arabidopsis* tissue. The data demonstrate that mRNAs encoding the enzymes of the pyrrolnitrin biosynthetic pathway accumulate in transgenic plants.

D. Cloning of Resorcinol Biosynthetic Genes from *Pseudomonas*

2-hexyl-5-propyl-resorcinol is a further APS produced by certain strains of *Pseudomonas*. It has been shown to have antipathogenic activity against Gram-positive bacteria (in particular *Clavibacter* spp.), mycobacteria, and fungi.

Example 13: Isolation of Genes Encoding Resorcinol

Two transposon-insertion mutants have been isolated which lack the ability to produce the antipathogenic substance 2-hexyl-5-propyl-resorcinol which is a further substance known to be under the global regulation of the *gafA* gene in *Pseudomonas fluorescens* (WO 94/01561). The insertion transposon TnCIB116 was used to generate libraries of mutants in MOCG134 and a *gafA*⁻ derivative of MOCG134 (BL1826). The former was screened for changes in fungal inhibition in vitro; the latter was screened for genes regulated by *gafA* after introduction of *gafA* on a plasmid (see Section C). Selected mutants were characterized by HPLC to assay for production of known compounds such as pyrrolnitrin and 2-hexyl-5-propyl-resorcinol. The HPLC assay enabled a comparison of the novel mutants to the wild-type parental strain. In each case, the HPLC peak corresponding to 2-hexyl-5-propyl-resorcinol was missing in the mutant. The mutant derived from MOCG134 is designated BL1846. The mutant derived from BL1826 is designated BL1911. HPLC for resorcinol follows the same procedure as for pyrrolnitrin (see example 11) except that 100% methanol is applied to the column at 20 min to elute resorcinol.

The resorcinol biosynthetic genes can be cloned from the above-identified mutants in the following manner. Genomic DNA is prepared from the mutants, and clones containing the transposon insertion and adjacent *Pseudomonas* sequence are obtained by selecting for kanamycin resistant clones (kanamycin resistance is encoded by the transposon). The

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cloned *Pseudomonas* sequence is then used as a probe to identify the native sequences from a genomic library of *P. fluorescens* MOCG134. The cloned native genes are likely to represent resorcinol biosynthetic genes.

E. Cloning Soraphen Biosynthetic Genes from *Sorangium*

Soraphen is a polyketide antibiotic produced by the myxobacterium *Sorangium cellulosum*. This compound has broad antifungal activities which make it useful for agricultural applications. In particular, soraphen has activity against a broad range of foliar pathogens.

Example 14: Isolation of the Soraphen Gene Cluster

Genomic DNA was isolated from *Sorangium cellulosum* and partially digested with *Sau3A*. Fragments of between 30 and 40 kb were size selected and cloned into the cosmid vector pHCT9 (Hohn & Collins, Gene 11: 291-298 (1980)) which had been previously digested with *BamHI* and treated with alkaline phosphatase to prevent self ligation. The cosmid library thus prepared was probed with a 4.6 kb fragment which contains the *gal* region of *Streptomyces violaceoruber* strain Tü22 encoding ORFs 1-4 responsible for the biosynthesis of granaticin in *S. violaceoruber*. Cosmid clones which hybridized to the *gal* probe were identified and DNA was prepared for analysis by restriction digestion and further hybridization. Cosmid p98/1 was identified to contain a 1.8 kb *Sall* fragment which hybridized strongly to the *gal* region; this *Sall* fragment was located within a larger 6.5 kb *PvuI* fragment within the ~40 kb insert of p98/1. Determination of the sequence of part of the 1.8 kb *Sall* insert revealed homology to the acetyltransferase proteins required for the synthesis of erythromycin. Restriction mapping of the cosmid p98/1 was undertaken and generated the map depicted in figure 7. A viable culture of E.coli HB101 comprising cosmid clone 98/1 has been deposited with the Agricultural Research Culture Collection (NRRL) at 1815 N. University Street, Peoria, Illinois 61604 U.S.A. on May 20, 1994, under the accession number NRRL B-21255. The DNA sequence of the soraphen gene cluster is disclosed in SEQ ID NO:6.

Example 15: Functional Analysis of the Soraphen Gene Cluster

The regions within p98/1 that encode proteins with a role in the biosynthesis of soraphen were identified through gene disruption experiments. Initially, DNA fragments were derived from cosmid p98/1 by restriction with *PvuI* and cloned into the unique *PvuI* cloning site

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(which is within the gene for ampicillin resistance) of the wide host-range plasmid pSUP2021 (Simon *et al.* in: Molecular Genetics of the Bacteria-Plant Interaction (ed.: A Puhler), Springer Verlag, Berlin pp 98-106 (1983)). Transformed *E. coli* HB101 was selected for resistance to chloramphenicol, but sensitivity to ampicillin. Selected colonies carrying appropriate inserts were transferred to *Sorangium cellulosum* SJ3 by conjugation using the method described in the published application EP 0 501 921 (to Ciba-Geigy). Plasmids were transferred to *E. coli* ED8767 carrying the helper plasmid pUZ8 (Hedges & Mathew, Plasmid 2: 269-278 (1979)) and the donor cells were incubated with *Sorangium cellulosum* SJ3 cells from a stationary phase culture for conjugative transfer essentially as described in EP 0 501 921 (example 5) and EP *the later app.* (example 2). Selection was on kanamycin, phleomycin and streptomycin. It has been determined that no plasmids tested thus far are capable of autonomous replication in *Sorangium cellulosum*, but rather, integration of the entire plasmid into the chromosome by homologous recombination occurs at a site within the cloned fragment at low frequency. These events can be selected for by the presence of antibiotic resistance markers on the plasmid. Integration of the plasmid at a given site results in the insertion of the plasmid into the chromosome and the concomitant disruption of this region from this event. Therefore, a given phenotype of interest, *i.e.* soraphen production, can be assessed, and disruption of the phenotype will indicate that the DNA region cloned into the plasmid must have a role in the determination of this phenotype.

Recombinant pSUP2021 clones with *PvuI* inserts of approximate size 6.5 kb (pSN105/7), 10 kb (pSN120/10), 3.8 kb (pSN120/43-39) and 4.0 kb (pSN120/46) were selected. The map locations (in kb) of these *PvuI* inserts as shown in Figure 7 are: pSN105/7 - 25.0-31.7, pSN120/10 - 2.5-14.5, pSN120/43-39 - 16.1-20.0, and pSN120/46 - 20.0-24.0. pSN105/7 was shown by digestion with *PvuI* and *Sall* to contain the 1.8 kb fragment referred to above in example 11. Gene disruptions with the 3.8, 4.0, 6.5, and 10 kb *PvuI* fragments all resulted in the elimination of soraphen production. These results indicate that all of these fragments contain genes or fragments of genes with a role in the production of this compound.

Subsequently gene disruption experiments were performed with two *BglII* fragments derived from cosmid p98/1. These were of size 3.2 kb (map location 32.4-35.6 on Figure 7) and 2.9

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kb (map location 35.6-38.5 on Figure 7). These fragments were cloned into the *Bam*HI site of plasmid pCIB132 that was derived from pSUP2021 according to Figure 8. The ~5 kb *Not*I fragment of pSUP2021 was excised and inverted, followed by the removal of the ~3kb *Bam*HI fragment. Neither of these *Bgl*II fragments was able to disrupt soraphen biosynthesis when reintroduced into *Sorangium* using the method described above. This indicates that the DNA of these fragments has no role in soraphen biosynthesis. Examination of the DNA sequence indicates the presence of a thioesterase domain 5' to, but near the *Bgl*II site at location 32.4. In addition, there are transcription stop codons immediately after the thioesterase domain which are likely to demarcate the end of the ORF1 coding region. As the 2.9 and 3.2 kb *Bgl*II fragments are immediately to the right of these sequences it is likely that there are no other genes downstream from ORF1 that are involved in soraphen biosynthesis.

Delineation of the left end of the biosynthetic region required the isolation of two other cosmid clones, pJL1 and pJL3, that overlap p98/1 on the left end, but include more DNA leftwards of p98/1. These were isolated by hybridization with the 1.3 kb *Bam*HI fragment on the extreme left end of p98/1 (map location 0.0-1.3) to the *Sorangium cellulosum* gene library. It should be noted that the *Bam*HI site at 0.0 does not exist in the *S. cellulosum* chromosome but was formed as an artifact from the ligation of a *Sau*3A restriction fragment derived from the *Sorangium cellulosum* genome into the *Bam*HI cloning site of pH7C9. Southern hybridization with the 1.3 kb *Bam*HI fragment demonstrated that pJL1 and pJL3 each contain an approximately 12.5 kb *Bam*HI fragment that contains sequences common to the 1.3 kb fragment as this fragment is in fact delineated by the *Bam*HI site at position 1.3. A viable culture of E.coli HB101 comprising cosmid clone pJL3 has been deposited with the Agricultural Research Culture Collection (NRRL) at 1815 N. University Street, Peoria, Illinois 61604 U.S.A. on May 20, 1994, under the accession number NRRL B-21254. Gene disruption experiments using the 12.5 kb *Bam*HI fragment indicated that this fragment contains sequences that are involved in the synthesis of soraphen. Gene disruption using smaller *Eco*RV fragments derived from this region indicated the requirement of this region for soraphen biosynthesis. For example, two *Eco*RV fragments of 3.4 and 1.1 kb located adjacent to the distal *Bam*HI site at the left end of the 12.5 kb fragment resulted in a reduction in soraphen biosynthesis when used in gene disruption experiments.

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Example 16: Sequence Analysis of the Soraphen Gene Cluster

The DNA sequence of the soraphen gene cluster was determined from the *PvuI* site at position 2.5 to the *BglII* site at position 32.4 (see Figure 7) using the Taq DyeDeoxy Terminator Cycle Sequencing Kit supplied by Applied Biosystems, Inc., Foster City, CA. following the protocol supplied by the manufacturer. Sequencing reactions were run on a Applied Biosystems 373A Automated DNA Sequencer and the raw DNA sequence was assembled and edited using the "INHERIT" software package also from Applied Biosystems, Inc.. The pattern recognition program "FRAMES" was used to search for open reading frames (ORFs) in all six translation frames of the DNA sequence. In total approximately 30 kb of contiguous DNA was assembled and this corresponds to the region determined to be critical to soraphen biosynthesis in the disruption experiments described in example 12. This sequence encodes two ORFs which have the structure described below.

ORF1:

ORF1 is approximately 25.5 kb in size and encodes five biosynthetic modules with homology to the modules found in the erythromycin biosynthetic genes of *Saccharopolyspora erythraea* (Donadio *et al.* Science 252: 675-679 (1991)). Each module contains a β -ketoacylsynthase (KS), an acyltransferase (AT), a ketoreductase (KR) and an acyl carrier protein (ACP) domain as well as β -ketone processing domains which may include a dehydratase (DH) and/or enoyl reductase (ER) domain. In the biosynthesis of the polyketide structure each module directs the incorporation of a new two carbon extender unit and the correct processing of the β -ketone carbon.

ORF2:

In addition to ORF1, DNA sequence data from the p98/1 fragment spanning the *PvuI* site at 2.5 kb and the *SmaI* site at 6.2 kb, indicated the presence of a further ORF (ORF2) immediately adjacent to ORF1. The DNA sequence demonstrates the presence of a typical biosynthetic module that appears to be encoded on an ORF whose 5' end is not yet sequenced and is some distance to the left. By comparison to other polyketide biosynthetic gene units and the number of carbon atoms in the soraphen ring structure it is likely that there should be a total of eight modules in order to direct the synthesis of 17 carbon molecule soraphen. Since there are five modules in ORF1 described above, it was predicted that ORF2 contains a further three and that these would extend beyond the left

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end of cosmid p98/1 (position 0 in Figure 7). This is entirely consistent with the gene description of example 12. The cosmid clones pJL1 and pJL3 extending beyond the left end of p98/1 presumably carry the sequence encoding the remaining modules required for soraphen biosynthesis.

Example 17: Soraphen: Requirement for Methylation

Synthesis of polyketides typically requires, as a first step, the condensation of a starter unit (commonly acetate) and an extender unit (malonate) with the loss of one carbon atom in the form of CO₂ to yield a three-carbon chain. All subsequent additions result in the addition of two carbon units to the polyketide ring (Donadio *et al.* Science 252: 675-679 (1991)). Since soraphen has a 17-carbons ring, it is likely that there are 8 biosynthetic modules required for its synthesis. Five modules are encoded in ORF1 and a sixth is present at the 3' end of ORF2. As explained above, it is likely that the remaining two modules are also encoded by ORF2 in the regions that are in the 15 kb *Bam*HI fragment from pJL1 and pJL3 for which the sequence has not yet been determined.

The polyketide modular biosynthetic apparatus present in *Sorangium cellulosum* is required for the production of the compound, soraphen C, which has no antipathogenic activity. The structure of this compound is the same as that of the antipathogenic soraphen A with the exception that the O-methyl groups of soraphen A at positions 6, 7, and 14 of the ring are hydroxyl groups. These are methylated by a specific methyltransferase to form the active compound soraphen A. A similar situation exists in the biosynthesis of erythromycin in *Saccharopolyspora erythraea*. The final step in the biosynthesis of this molecule is the methylation of three hydroxyl groups by a methyltransferase (Haydock *et al.*, Mol. Gen. Genet. 230: 120-128 (1991)). It is highly likely, therefore, that a similar methyltransferase (or possibly more than one) operates in the biosynthesis of soraphen A (soraphen C is unmethylated and soraphen B is partially methylated). In all polyketide biosynthesis systems examined thus far, all of the biosynthetic genes and associated methylases are clustered together (Summers *et al.* J Bacteriol 174: 1810-1820 (1992)). It is also probable, therefore, that a similar situation exists in the soraphen operon and that the gene encoding the methyltransferase/s required for the conversion of soraphen B and C to soraphen A is located near the ORF1 and ORF2 that encode the polyketide synthase. The results of the gene disruption experiments described above indicate that this gene is not located

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immediately downstream from the 3' end of ORF1 and that it is likely located upstream of ORF2 in the DNA contained in pJL1 and pJL3. Thus, using standard techniques in the art, the methyltransferase gene can be cloned and sequenced.

Soraphen Determination

Sorangium cellulosum cells were cultured in a liquid growth medium containing an exchange resin, XAD-5 (Rohm and Haas) (5% w/v). The soraphen A produced by the cells bound to the resin which was collected by filtration through a polyester filter (Sartorius B 420-47-N) and the soraphen was released from the resin by extraction with 50 ml isopropanol for 1 hr at 30 C. The isopropanol containing soraphen A was collected and concentrated by drying to a volume of approximately 1 ml. Aliquots of this sample were analyzed by HPLC at 210 nm to detect and quantify the soraphen A. This assay procedure is specific for soraphen A (fully methylated); partially and non-methylated soraphen forms have a different R_T and are not measured by this procedure. This procedure was used to assay soraphen A production after gene disruption.

F. Cloning and Characterization of Phenazine Biosynthetic Genes from *Pseudomonas aureofaciens*

The phenazine antibiotics are produced by a variety of *Pseudomonas* and *Streptomyces* species as secondary metabolites branching off the shikimic acid pathway. It has been postulated that two chorismic acid molecules are condensed along with two nitrogens derived from glutamine to form the three-ringed phenazine pathway precursor phenazine-1,6-dicarboxylate. However, there is also genetic evidence that anthranilate is an intermediate between chorismate and phenazine-1,6-dicarboxylate (Essar *et al.*, J. Bacteriol. 172: 853-866 (1990)). In *Pseudomonas aureofaciens* 30-84, production of three phenazine antibiotics, phenazine-1-carboxylic acid, 2-hydroxyphenazine-1-carboxylic acid, and 2-hydroxyphenazine, is the major mode of action by which the strain protects wheat from the fungal phytopathogen *Gaeumannomyces graminis* var. *tritici* (Pierson & Thomashow, MPMI 5: 330-339 (1992)). Likewise, in *Pseudomonas fluorescens* 2-79, phenazine production is a major factor in the control of *G. graminis* var. *tritici* (Thomashow & Weller, J. Bacteriol. 170: 3499-3508 (1988)).

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Example 18: Isolation of the Phenazine Biosynthetic Genes

Pierson & Thomashow (*supra*) have previously described the cloning of a cosmid which confers a phenazine biosynthesis phenotype on transposon insertion mutants of *Pseudomonas aureofaciens* strain 30-84 which were disrupted in their ability to synthesize phenazine antibiotics. A mutant library of strain 30-84 was made by conjugation with *E. coli* S17-1(pSUP1021) and mutants unable to produce phenazine antibiotics were selected. Selected mutants were unable to produce phenazine carboxylic acid, 2-hydroxyphenazine or 2-hydroxy-phenazine carboxylic acid. These mutants were transformed by a cosmid genomic library of strain 30-84 leading to the isolation of cosmid pLSP259 which had the ability to complement phenazine mutants by the synthesis of phenazine carboxylic acid, 2-hydroxyphenazine and 2-hydroxy-phenazinecarboxylic acid. pLSP259 was further characterized by transposon mutagenesis using the λ ::Tn5 phage described by de Bruijn & Lupski (Gene 27: 131-149 (1984)). Thus a segment of approximately 2.8 kb of DNA was identified as being responsible for the phenazine complementing phenotype; this 2.8 kb segment is located within a larger 9.2 kb *EcoRI* fragment of pLSP259. Transfer of the 9.2 kb *EcoRI* fragment and various deletion derivatives thereof to *E. coli* under the control of the *lacZ* promoter was undertaken to assay for the production in *E. coli* of phenazine. The shortest deletion derivative which was found to confer biosynthesis of all three phenazine compounds to *E. coli* contained an insert of approximately 6 kb and was designated pLSP18-6H3del3. This plasmid contained the 2.8 kb segment previously identified as being critical to phenazine biosynthesis in the host 30-84 strain and was provided by Dr LS Pierson (Department of Plant Pathology, U Arizona, Tucson, AZ) for sequence characterization. Other deletion derivatives were able to confer production of phenazine-carboxylic acid on *E. coli*, without the accompanying production of 2-hydroxyphenazine and 2-hydroxyphenazinecarboxylic acid suggesting that at least two genes might be involved in the synthesis of phenazine and its hydroxy derivatives.

The DNA sequence comprising the genes for the biosynthesis of phenazine is disclosed in SEQ ID NO:17. Plasmid pCIB3350 contains the PstI-HindIII fragment of the phenazine gene cluster and has been deposited with the Agricultural Research Culture Collection (NRRL) at 1815 N. University Street, Peoria, Illinois 61604 U.S.A. on May 20, 1994, under the accession number NRRL B-21257. Plasmid pCIB3351 contains the EcoRI-PstI fragment of the phenazine gene cluster and has been deposited with the Agricultural Research Culture

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Collection (NRRL) at 1815 N. University Street, Peoria, Illinois 61604 U.S.A. on May 20, 1994, under the accession number NRRL B-21258. pCIB3350 along with pCIB3351 comprises the entire phenazine gene of SEQ ID NO:17. Determination of the DNA sequence of the insert of pLSP18-6H3del3 revealed the presence of four ORFs within and adjacent to the critical 2.8 kb segment. ORF1 (SEQ ID NO:18) was designated *phz1*, ORF2 (SEQ ID NO:19) was designated *phz2*, and ORF3 (SEQ ID NO:20) was designated *phz3*, and ORF4 (SEQ ID NO:22) was designated *phz4*. The DNA sequence of *phz4* is shown in SEQ ID NO:21. *phz1* is approximately 1.35 kb in size and has homology at the 5' end to the *entB* gene of *E. coli*, which encodes isochorismatase. *phz2* is approximately 1.15 kb in size and has some homology at the 3' end to the *trpG* gene which encodes the beta subunit of anthranilate synthase. *phz3* is approximately 0.85 kb in size. *phz4* is approximately 0.65 kb in size and is homologous to the *pdxH* gene of *E. coli* which encodes pyridoxamine 5'-phosphate oxidase.

Phenazine Determination

Thomashow *et al.* (Appl Environ Microbiol **56**: 908-912 (1990)) describe a method for the isolation of phenazine. This involves acidifying cultures to pH 2.0 with HCl and extraction with benzene. Benzene fractions are dehydrated with Na₂SO₄ and evaporated to dryness. The residue is redissolved in aqueous 5% NaHCO₃, reextracted with an equal volume of benzene, acidified, partitioned into benzene and redried. Phenazine concentrations are determined after fractionation by reverse-phase HPLC as described by Thomashow *et al.* (*supra*).

G. Cloning Peptide Antipathogenic Genes

This group of substances is diverse and is classifiable into two groups: (1) those which are synthesized by enzyme systems without the participation of the ribosomal apparatus, and (2) those which require the ribosomally-mediated translation of an mRNA to provide the precursor of the antibiotic.

Non-Ribosomal Peptide Antibiotics.

Non-Ribosomal Peptide Antibiotics are assembled by large, multifunctional enzymes which activate, modify, polymerize and in some cases cyclize the subunit amino acids, forming

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polypeptide chains. Other acids, such as aminoadipic acid, diaminobutyric acid, diaminopropionic acid, dihydroxyamino acid, isoserine, dihydroxybenzoic acid, hydroxyisovaleric acid, (4R)-4-[(E)-2-butenyl]-4,N-dimethyl-L-threonine, and ornithine are also incorporated (Katz & Demain, *Bacteriological Review* 41: 449-474 (1977); Kleinkauf & von Dohren, *Annual Review of Microbiology* 41: 259-289 (1987)). The products are not encoded by any mRNA, and ribosomes do not directly participate in their synthesis. Peptide antibiotics synthesized non-ribosomally can in turn be grouped according to their general structures into linear, cyclic, lactone, branched cyclopeptide, and depsipeptide categories (Kleinkauf & von Dohren, *European Journal of Biochemistry* 192: 1-15 (1990)). These different groups of antibiotics are produced by the action of modifying and cyclizing enzymes; the basic scheme of polymerization is common to them all. Non-ribosomally synthesized peptide antibiotics are produced by both bacteria and fungi, and include edeine, linear gramicidin, tyrocidine and gramicidin S from *Bacillus brevis*, mycobacillin from *Bacillus subtilis*, polymyxin from *Bacillus polymyxa*, etamycin from *Streptomyces griseus*, echinomycin from *Streptomyces echinatus*, actinomycin from *Streptomyces clavuligerus*, enterochelin from *Escherichia coli*, gamma-(alpha-L-aminoadipyl)-L-cysteinyl-D-valine (ACV) from *Aspergillus nidulans*, alamethicine from *Trichoderma viride*, destruxin from *Metarhizium anisopliae*, enniatin from *Fusarium oxysporum*, and beauvericin from *Beauveria bassiana*. Extensive functional and structural similarity exists between the prokaryotic and eukaryotic systems, suggesting a common origin for both. The activities of peptide antibiotics are similarly broad, toxic effects of different peptide antibiotics in animals, plants, bacteria, and fungi are known (Hansen, *Annual Review of Microbiology* 47: 535-564 (1993); Katz & Demain, *Bacteriological Reviews* 41: 449-474 (1977); Kleinkauf & von Dohren, *Annual Review of Microbiology* 41: 259-289 (1987); Kleinkauf & von Dohren, *European Journal of Biochemistry* 192: 1-15 (1990); Kolter & Moreno, *Annual Review of Microbiology* 46: 141-163 (1992)).

Amino acids are activated by the hydrolysis of ATP to form an adenylated amino or hydroxy acid, analogous to the charging reactions carried out by aminoacyl-tRNA synthetases, and then covalent thioester intermediates are formed between the amino acids and the enzyme(s), either at specific cysteine residues or to a thiol donated by pantetheine. The amino acid-dependent hydrolysis of ATP is often used as an assay for peptide antibiotic enzyme complexes (Ishihara, *et al.*, *Journal of Bacteriology* 171: 1705-1711 (1989)). Once

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bound to the enzyme, activated amino acids may be modified before they are incorporated into the polypeptide. The most common modifications are epimerization of L-amino (hydroxy) acids to the D- form, N-acylations, cyclizations and N-methylations. Polymerization occurs through the participation of a pantetheine cofactor, which allows the activated subunits to be sequentially added to the polypeptide chain. The mechanism by which the peptide is released from the enzyme complex is important in the determination of the structural class in which the product belongs. Hydrolysis or aminolysis by a free amine of the thiolester will yield a linear (unmodified or terminally aminated) peptide such as edeine; aminolysis of the thiolester by amine groups on the peptide itself will give either cyclic (attack by terminal amine), such as gramicidin S, or branched (attack by side chain amine), such as bacitracin, peptides; lactonization with a terminal or side chain hydroxy will give a lactone, such as destruxin, branched lactone, or cyclodepsipeptide, such as beauvericin.

The enzymes which carry out these reactions are large multifunctional proteins, having molecular weights in accord with the variety of functions they perform. For example, gramicidin synthetases 1 and 2 are 120 and 280 kDa, respectively; ACV synthetase is 230 kDa; enniatin synthetase is 250 kDa; bacitracin synthetases 1, 2, 3 are 335, 240, and 380 kDa, respectively (Katz & Demain, *Bacteriological Reviews* **41**: 449-474 (1977); Kleinkauf & von Dohren, *Annual Review of Microbiology* **41**: 259-289 (1987); Kleinkauf & von Dohren, *European Journal of Biochemistry* **192**: 1-15 (1990)). The size and complexity of these proteins means that relatively few genes must be cloned in order for the capability for the complete nonribosomal synthesis of peptide antibiotics to be transferred. Further, the functional and structural homology between bacterial and eukaryotic synthetic systems indicates that such genes from any source of a peptide antibiotic can be cloned using the available sequence information, current functional information, and conventional microbiological techniques. The production of a fungicidal, insecticidal, or bactericidal peptide antibiotic in a plant is expected to produce an advantage with respect to the resistance to agricultural pests.

Example 19: Cloning of Gramicidin S Bi synthesis Genes

Gramicidin S is a cyclic antibiotic peptide and has been shown to inhibit the germination of fungal spores (Murray, *et al.*, *Letters in Applied Microbiology* **3**: 5-7 (1986)), and may

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therefore be useful in the protection of plants against fungal diseases. The gramicidin S biosynthesis operon (*grs*) from *Bacillus brevis* ATCC 9999 has been cloned and sequenced, including the entire coding sequences for gramicidin synthetase 1 (GS1, *grsA*), another gene in the operon of unknown function (*grsT*), and GS2 (*grsB*) (Kratzschmar, *et al.*, Journal of Bacteriology 171: 5422-5429 (1989); Krause, *et al.*, Journal of Bacteriology 162: 1120-1125 (1985)). By methods well known in the art, pairs of PCR primers are designed from the published DNA sequence which are suitable for amplifying segments of approximately 500 base pairs from the *grs* operon using isolated *Bacillus brevis* ATCC 9999 DNA as a template. The fragments to be amplified are (1) at the 3' end of the coding region of *grsB*, spanning the termination codon, (2) at the 5' end of the *grsB* coding sequence, including the initiation codon, (3) at the 3' end of the coding sequence of *grsA*, including the termination codon, (4) at the 5' end of the coding sequence of *grsA*, including the initiation codon, (5) at the 3' end of the coding sequence of *grsT*, including the termination codon, and (6) at the 5' end of the coding sequence of *grsT*, including the initiation codon. The amplified fragments are radioactively or nonradioactively labeled by methods known in the art and used to screen a genomic library of *Bacillus brevis* ATCC 9999 DNA constructed in a vector such as λ EMBL3. The 6 amplified fragments are used in pairs to isolate cloned fragments of genomic DNA which contain intact coding sequences for the three biosynthetic genes. Clones which hybridize to probes 1 and 2 will contain an intact *grsB* sequence, those which hybridize to probes 3 and 4 will contain an intact *grsA* gene, those which hybridize to probes 5 and 6 will contain an intact *grsT* gene. The cloned *grsA* is introduced into *E. coli* and extracts prepared by lysing transformed bacteria through methods known in the art are tested for activity by the determination of phenylalanine-dependent ATP-PP_i exchange (Krause, *et al.*, Journal of Bacteriology 162: 1120-1125 (1985)) after removal of proteins smaller than 120 kDa by gel filtration chromatography. *GrsB* is tested similarly by assaying gel-filtered extracts from transformed bacteria for proline, valine, ornithine and leucine-dependent ATP-PP_i exchange.

Example 20: Cloning of Penicillin Biosynthesis Genes

A 38 kb fragment of genomic DNA from *Penicillium chrysogenum* transfers the ability to synthesize penicillin to fungi, *Aspergillus niger*, and *Neurospora crassa*, which do not normally produce it (Smith, *et al.*, Bio/Technology 8: 39-41 (1990)). The genes which are responsible for biosynthesis, delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase,

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isopenicillin N synthetase, and isopenicillin N acyltransferase have been individually cloned from *P. chrysogenum* and *Aspergillus nidulans*, and their sequences determined (Ramon, *et al.*, Gene 57: 171-181 (1987); Smith, *et al.*, EMBO Journal 9: 2743-2750 (1990); Tobin, *et al.*, Journal of Bacteriology 172: 5908-5914 (1990)). The cloning of these genes is accomplished by following the PCR-based approach described above to obtain probes of approximately 500 base pairs from genomic DNA from either *Penicillium chrysogenum* (for example, strain AS-P-78, from Antibioticos, S.A., Leon, Spain), or from *Aspergillus nidulans* for example, strain G69. Their integrity and function may be checked by transforming the non-producing fungi listed above and assaying for antibiotic production and individual enzyme activities as described (Smith, *et al.*, Bio/Technology 8: 39-41 (1990)).

Example 21: Cloning of Bacitracin A Biosynthesis Genes

Bacitracin A is a branched cyclopeptide antibiotic which has potential for the enhancement of disease resistance to bacterial plant pathogens. It is produced by *Bacillus licheniformis* ATCC 10716, and three multifunctional enzymes, bacitracin synthetases (BA) 1, 2, and 3, are required for its synthesis. The molecular weights of BA1, BA2, and BA3 are 335 kDa, 240 kDa, and 380 kDa, respectively. A 32 kb fragment of *Bacillus licheniformis* DNA which encodes the BA2 protein and part of the BA3 protein shows that at least these two genes are linked (Ishihara, *et al.*, Journal of Bacteriology 171: 1705-1711 (1989)). Evidence from gramicidin S, penicillin, and surfactin biosynthetic operons suggest that the first protein in the pathway, BA1, will be encoded by a gene which is relatively close to BA2 and BA3. BA3 is purified by published methods, and it is used to raise an antibody in rabbits (Ishihara, *et al. supra*). A genomic library of *Bacillus licheniformis* DNA is transformed into *E. coli* and clones which express antigenic determinants related to BA3 are detected by methods known in the art. Because BA1, BA2, and BA3 are antigenically related, the detection method will provide clones encoding each of the three enzymes. The identity of each clone is confirmed by testing extracts of transformed *E. coli* for the appropriate amino acid-dependent ATP-PP_i exchange. Clones encoding BA1 will exhibit leucine-, glutamic acid-, and isoleucine-dependent ATP-PP_i exchange, those encoding BA2 will exhibit lysine- and ornithine-dependent exchange, and those encoding BA3 will exhibit isoleucine, phenylalanine-, histidine-, aspartic acid-, and asparagine-dependent exchange. If one or two genes are obtained by this method, the others are isolated by techniques known in the art as "walking"

or "chromosome walking" techniques (Sambrook et al, in: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989).

Example 22: Cloning of Beauvericin and Destruxin Biosynthesis Genes

Beauvericin is an insecticidal hexadepsipeptide produced by the fungus *Beauveria bassiana* (Kleinkauf & von Dohren, European Journal of Biochemistry 192: 1-15 (1990)) which will provide protection to plants from insect pests. It is an analog of enniatin, a phytotoxic hexadepsipeptide produced by some phytopathogenic species of *Fusarium* (Burmeister & Plattner, Phytopathology 77: 1483-1487 (1987)). Destruxin is an insecticidal lactone peptide produced by the fungus *Metarhizium anisopliae* (James, et al., Journal of Insect Physiology 39: 797-804 (1993)). Monoclonal antibodies directed to the region of the enniatin synthetase complex responsible for N-methylation of activated amino acids cross react with the synthetases for beauvericin and destruxin, demonstrating their structural relatedness (Kleinkauf & von Dohren, European Journal of Biochemistry 192: 1-15 (1990)). The gene for enniatin synthetase gene (*esyn1*) from *Fusarium scirpi* has been cloned and sequenced (Haese, et al., Molecular Microbiology 7: 905-914 (1993)), and the sequence information is used to carry out a cloning strategy for the beauvericin synthetase and destruxin synthetase genes as described above. Probes for the beauvericin synthetase (BE) gene and the destruxin synthetase (DXS) gene are produced by amplifying specific regions of *Beauveria bassiana* genomic DNA or *Metarhizium anisopliae* genomic DNA using oligomers whose sequences are taken from the enniatin synthetase sequence as PCR primers. Two pairs of PCR primers are chosen, with one pair capable of causing the amplification of the segment of the BE gene spanning the initiation codon, and the other pair capable of causing the amplification of the segment of the BE gene which spans the termination codon. Each pair will cause the production of a DNA fragment which is approximately 500 base pairs in size. Library of genomic DNA from *Beauveria bassiana* and *Metarhizium anisopliae* are probed with the labeled fragments, and clones which hybridize to both of them are chosen. Complete coding sequences of beauvericin synthetase will cause the appearance of phenylalanine-dependent ATP-PP_i exchange in an appropriate host, and that of destruxin will cause the appearance of valine-, isoleucine-, and alanine-dependent ATP-PP_i exchange. Extracts from these transformed organisms will also carry out the cell-free biosynthesis of beauvericin and destruxin, respectively.

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Example 23: Cloning genes for the Biosynthesis of an Unknown Peptide Antibiotic

The genes for any peptide antibiotic are cloned by the use of conserved regions within the coding sequence. The functions common to all peptide antibiotic synthetases, that is, amino acid activation, ATP-, and pantotheine binding, are reflected in a repeated domain structure in which each domain spans approximately 600 amino acids. Within the domains, highly conserved sequences are known, and it is expected that related sequences will exist in any peptide antibiotic synthetase, regardless of its source. The published DNA sequences of peptide synthetase genes, including gramicidin synthetases 1 and 2 (Hori, *et al.*, *Journal of Biochemistry* 106: 639-645 (1989); Krause, *et al.*, *Journal of Bacteriology* 162: 1120-1125 (1985); Turgay, *et al.*, *Molecular Microbiology* 6: 529-546 (1992)), tyrocidine synthetase 1 and 2 (Weckermann, *et al.*, *Nucleic Acids Research* 16: 11841 (1988)), ACV synthetase (MacCabe, *et al.*, *Journal of Biological Chemistry* 266: 12646-12654 (1991)), enniatin synthetase (Haese, *et al.*, *Molecular Microbiology* 7: 905-914 (1993)), and surfactin synthetase (Fuma, *et al.*, *Nucleic Acids Research* 21: 93-97 (1993); Grandi, *et al.*, Eleventh International Spores Conference (1992)) are compared and the individual repeated domains are identified. The domains from all the synthetases are compared as a group, and the most highly conserved sequences are identified. From these conserved sequences, DNA oligomers are designed which are suitable for hybridizing to all of the observed variants of the sequence, and another DNA sequence which lies, for example, from 0.1 to 2 kilobases away from the first DNA sequence, is used to design another DNA oligomer. Such pairs of DNA oligomers are used to amplify by PCR the intervening segment of the unknown gene by combining them with genomic DNA prepared from the organism which produces the antibiotic, and following a PCR amplification procedure. The fragment of DNA which is produced is sequenced to confirm its identity, and used as a probe to identify clones containing larger segments of the peptide synthetase gene in a genomic library. A variation of this approach, in which the oligomers designed to hybridize to the conserved sequences in the genes were used as hybridization probes themselves, rather than as primers of PCR reactions, resulted in the identification of part of the surfactin synthetase gene from *Bacillus subtilis* ATCC 21332 (Borchert, *et al.*, *FEMS Microbiological Letters* 92: 175-180 (1992)). The cloned genomic DNA which hybridizes to the PCR-generated probe is sequenced, and the complete coding sequence is obtained by "walking" procedures. Such "walking" procedures will also yield other genes required for the peptide antibiotic synthesis, because they are known to be clustered.

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Another method of obtaining the genes which code for the synthetase(s) of a novel peptide antibiotic is by the detection of antigenic determinants expressed in a heterologous host after transformation with an appropriate genomic library made from DNA from the antibiotic-producing organism. It is expected that the common structural features of the synthetases will be evidenced by cross-reactions with antibodies raised against different synthetase proteins. Such antibodies are raised against peptide synthetases purified from known antibiotic-producing organisms by known methods (Ishihara, *et al.*, *Journal of Bacteriology* 171: 1705-1711 (1989)). Transformed organisms bearing fragments of genomic DNA from the producer of the unknown peptide antibiotic are tested for the presence of antigenic determinants which are recognized by the anti-peptide synthetase antisera by methods known in the art. The cloned genomic DNA carried by cells which are identified by the antisera are recovered and sequenced. "Walking" techniques, as described earlier, are used to obtain both the entire coding sequence and other biosynthetic genes.

Another method of obtaining the genes which code for the synthetase of an unknown peptide antibiotic is by the purification of a protein which has the characteristics of the appropriate peptide synthetase, and determining all or part of its amino acid sequence. The amino acids present in the antibiotic are determined by first purifying it from a chloroform extract of a culture of the antibiotic-producing organism, for example by reverse phase chromatography on a C₁₈ column in an ethanol-water mixture. The composition of the purified compound is determined by mass spectrometry, NMR, and analysis of the products of acid hydrolysis. The amino or hydroxy acids present in the peptide antibiotic will produce ATP-PP_i exchange when added to a peptide-synthetase-containing extract from the antibiotic-producing organism. This reaction is used as an assay to detect the presence of the peptide synthetase during the course of a protein purification scheme, such as are known in the art. A substantially pure preparation of the peptide synthetase is used to determine its amino acid sequence, either by the direct sequencing of the intact protein to obtain the N-terminal amino acid sequence, or by the production, purification, and sequencing of peptides derived from the intact peptide synthetase by the action of specific proteolytic enzymes, as are known in the art. A DNA sequence is inferred from the amino acid sequence of the synthetase, and DNA oligomers are designed which are capable of hybridizing to such a coding sequence. The oligomers are used to probe a genomic library

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made from the DNA of the antibiotic-producing organism. Selected clones are sequenced to identify them, and complete coding sequences and associated genes required for peptide biosynthesis are obtained by using "walking" techniques. Extracts from organisms which have been transformed with the entire complement of peptide biosynthetic genes, for example bacteria or fungi, will produce the peptide antibiotic when provided with the required amino or hydroxy acids, ATP, and pantetheine.

Further methods appropriate for the cloning of genes required for the synthesis of non-ribosomal peptide antibiotics are described in Section B of the examples.

Ribosomally-Synthesized Peptide Antibiotics.

Ribosomally-Synthesized Peptide Antibiotics are characterized by the existence of a structural gene for the antibiotic itself, which encodes a precursor that is modified by specific enzymes to create the mature molecule. The use of the general protein synthesis apparatus for peptide antibiotic synthesis opens up the possibility for much longer polymers to be made, although these peptide antibiotics are not necessarily very large. In addition to a structural gene, further genes are required for extracellular secretion and immunity, and these genes are believed to be located close to the structural gene, in most cases probably on the same operon. Two major groups of peptide antibiotics made on ribosomes exist: those which contain the unusual amino acid lanthionine, and those which do not. Lanthionine-containing antibiotics (lantibiotics) are produced by gram-positive bacteria, including species of *Lactococcus*, *Staphylococcus*, *Streptococcus*, *Bacillus*, and *Streptomyces*. Linear lantibiotics (for example, nisin, subtilin, epidermin, and gallidermin), and circular lantibiotics (for example, duramycin and cinnamycin), are known (Hansen, Annual Review of Microbiology 47: 535-564 (1993); Kolter & Moreno, Annual Review of Microbiology 46: 141-163 (1992)). Lantibiotics often contain other characteristic modified residues such as dehydroalanine (DHA) and dehydrobutyrine (DHB), which are derived from the dehydration of serine and threonine, respectively. The reaction of a thiol from cysteine with DHA yields lanthionine, and with DHB yields β -methyllanthionine. Peptide antibiotics which do not contain lanthionine may contain other modifications, or they may consist only of the ordinary amino acids used in protein synthesis. Non-lanthionine-containing peptide antibiotics are produced by both gram-positive and gram-negative bacteria, including *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Enterococcus*, and

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Escherichia. Antibiotics in this category include lactacins, lactocins, sakacin A, pediocins, diplococcin, lactococcins, and microcins (Hansen, *supra*; Kolter & Moreno, *supra*). In general, peptide antibiotics whose synthesis is begun on ribosomes are subject to several types of post-translational processing, including proteolytic cleavage and modification of amino acid side chains, and require the presence of a specific transport and/or immunity mechanism. The necessity for protection from the effects of these antibiotics appears to contrast strongly with the lack of such systems for nonribosomal peptide antibiotics. This may be rationalized by considering that the antibiotic activity of many ribosomally-synthesized peptide antibiotics is directed at a narrow range of bacteria which are fairly closely related to the producing organism. In this situation, a particular method of distinguishing the producer from the competitor is required, or else the advantage is lost. As antibiotics, this property has limited the usefulness of this class of molecules for situations in which a broad range of activity is desirable, but enhances their attractiveness in cases when a very limited range of activities is advantageous. In eukaryotic systems, which are not known to be sensitive to any of this type of peptide antibiotic, it is not clear if production of a ribosomally-synthesized peptide antibiotic necessitates one of these transport systems, or if transport out of the cell is merely a matter of placing the antibiotic in a better location to encounter potential pathogens. This question can be addressed experimentally, as shown in the examples which follow.

Example 24: Cloning Genes for the Biosynthesis of a Lantibiotic

Examination of genes linked to the structural genes for the lantibiotics nisin, subtilin, and epidermin show several open reading frames which share sequence homology, and the predicted amino acid sequences suggest functions which are necessary for the maturation and transport of the antibiotic. The *spa* genes of *Bacillus subtilis* ATCC 6633, including *spaS*, the structural gene encoding the precursor to subtilin, have been sequenced (Chung & Hansen, *Journal of Bacteriology* 174: 6699-6702 (1992); Chung, *et al.*, *Journal of Bacteriology* 174: 1417-1422 (1992); Klein, *et al.*, *Applied and Environmental Microbiology* 58: 132-142 (1992)). Open reading frames were found only upstream of *spaS*, at least within a distance of 1-2 kilobases. Several of the open reading frames appear to part of the same transcriptional unit, *spaE*, *spaD*, *spaB*, and *spaC*, with a putative promoter upstream of *spaE*. Both *spaB*, which encodes a protein of 599 amino acids, and *spaD*, which encodes a protein of 177 amino acids, share homology to genes required for the transport

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of hemolysin, coding for the HylB and HlyD proteins, respectively. *SpaE*, which encodes a protein of 851 amino acids, is homologous to *nisB*, a gene linked to the structural gene for nisin, for which no function is known. *SpaC* codes for a protein of 442 amino acids of unknown function, but disruption of it eliminates production of subtilin. These genes are contained on a segment of genomic DNA which is approximately 7 kilobases in size (Chung & Hansen, *Journal of Bacteriology* 174: 6699-6702 (1992); Chung, *et al.*, *Journal of Bacteriology* 174: 1417-1422 (1992); Klein, *et al.*, *Applied and Environmental Microbiology* 58: 132-142 (1992)). It has not been clearly demonstrated if these genes are completely sufficient to confer the ability to produce subtilin. A 13.5 kilobasepair (kb) fragment from plasmid Tü32 of *Staphylococcus epidermis* Tü3298 containing the structural gene for epidermin (*epiA*), also contains five open reading frames denoted *epiA*, *epiB*, *epiC*, *epiD*, *epiQ*, and *epiP*. The genes *epiBC* are homologous to the genes *spaBC*, while *epiQ* appears to be involved in the regulation of the expression of the operon, and *epiP* may encode a protease which acts during the maturation of pre-epidermin to epidermin. *EpiD* encodes a protein of 181 amino acids which binds the coenzyme flavin mononucleotide, and is suggested to perform post-translational modification of pre-epidermin (Kupke, *et al.*, *Journal of Bacteriology* 174: (1992); Peschel, *et al.*, *Molecular Microbiology* 9: 31-39 (1993); Schnell, *et al.*, *European Journal of Biochemistry* 204: 57-68 (1992)). It is expected that many, if not all, of the genes required for the biosynthesis of a lantibiotic will be clustered, and physically close together on either genomic DNA or on a plasmid, and an approach which allows one of the necessary genes to be located will be useful in finding and cloning the others. The structural gene for a lantibiotic is cloned by designing oligonucleotide probes based on the amino acid sequence determined from a substantially purified preparation of the lantibiotic itself, as has been done with the lantibiotics lacticin 481 from *Lactococcus lactis* subsp. *lactis* CNRZ 481 (Piard, *et al.*, *Journal of Biological Chemistry* 268: 16361-16368 (1993)), streptococcin A-FF22 from *Streptococcus pyogenes* FF22 (Hynes, *et al.*, *Applied and Environmental Microbiology* 59: 1969-1971 (1993)), and salivaricin A from *Streptococcus salivarius* 203P (Ross, *et al.*, *Applied and Environmental Microbiology* 59: 2014-2021 (1993)). Fragments of bacterial DNA approximately 10-20 kilobases in size containing the structural gene are cloned and sequenced to determine regions of homology to the characterized genes in the *spa*, *epi*, and *nis* operons. Open reading frames which have homology to any of these genes or which lie in the same transcriptional unit as open reading frames having homology to any of these genes are

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cloned individually using techniques known in the art. A fragment of DNA containing all of the associated reading frames and no others is transformed into a non-producing strain of bacteria, such as *Escherichia coli*, and the production of the lantibiotic analyzed, in order to demonstrate that all the required genes are present.

Example 25: Cloning Genes for the Biosynthesis of a Non-Lanthionine Containing, Ribosomally Synthesized Peptide Antibiotic

The lack of the extensive modifications present in lantibiotics is expected to reduce the number of genes required to account for the complete synthesis of peptide antibiotics exemplified by lactacin F, sakacin A, lactococcin A, and helveticin J. Clustered genes involved in the biosynthesis of antibiotics were found in *Lactobacillus johnsonii* VPI11088, for lactacin F (Fremaux, *et al.*, Applied and Environmental Microbiology 59: 3906-3915 (1993)), in *Lactobacillus sake* Lb706 for sakacin A (Axelsson, *et al.*, Applied and Environmental Microbiology 59: 2868-2875 (1993)), in *Lactococcus lactis* for lactococcin A (Stoddard, *et al.*, Applied and Environmental Microbiology 58: 1952-1961 (1992)), and in *Pediococcus acidilactici* for pediocin PA-1 (Marugg, *et al.*, Applied and Environmental Microbiology, 58: 2360-2367 (1992)). The genes required for the biosynthesis of a novel non-lanthionine-containing peptide antibiotic are cloned by first determining the amino acid sequence of a substantially purified preparation of the antibiotic, designing DNA oligomers based on the amino acid sequence, and probing a DNA library constructed from either genomic or plasmid DNA from the producing bacterium. Fragments of DNA of 5-10 kilobases which contain the structural gene for the antibiotic are cloned and sequenced. Open reading frames which have homology to *sakB* from *Lactobacillus sake*, or to *lafX*, *ORFY*, or *ORFZ* from *Lactobacillus johnsonii*, or which are part of the same transcriptional unit as the antibiotic structural gene or genes having homology to those genes previously mentioned are individually cloned by methods known in the art. A fragment of DNA containing all of the associated reading frames and no others is transformed into a non-producing strain of bacteria, such as *Escherichia coli*, and the production of the antibiotic analyzed, in order to demonstrate that all the required genes are present.

H. Expression of Antibiotic Biosynthetic Genes in Microbial Hosts

Example 26: Overexpression of APS Biosynthetic Genes for Overproduction of APS using Fermentation-Type Technology

The APS biosynthetic genes of this invention can be expressed in heterologous organisms for the purposes of their production at greater quantities than might be possible from their native hosts. A suitable host for heterologous expression is *E. coli* and techniques for gene expression in *E. coli* are well known. For example, the cloned APS genes can be expressed in *E. coli* using the expression vector pKK223 as described in example 11. The cloned genes can be fused in transcriptional fusion, so as to use the available ribosome binding site cognate to the heterologous gene. This approach facilitates the expression of operons which encode more than one open reading frame as translation of the individual ORFs will thus be dependent on their cognate ribosome binding site signals. Alternatively APS genes can be fused to the vector's ATG (e.g. as an *NcoI* fusion) so as to use the *E. coli* ribosome binding site. For multiple ORF expression in *E. coli* (e.g. in the case of operons with multiple ORFs) this type of construct would require a separate promoter to be fused to each ORF. It is possible, however, to fuse the first ATG of the APS operon to the *E. coli* ribosome binding site while requiring the other ORFs to utilize their cognate ribosome binding sites. These types of construction for the overexpression of genes in *E. coli* are well known in the art. Suitable bacterial promoters include the *lac* promoter, the *tac* (*trp/lac*) promoter, and the P λ promoter from bacteriophage λ . Suitable commercially available vectors include, for example, pKK223-3, pKK233-2, pDR540, pDR720, pYEJ001 and pPL-Lambda (from Pharmacia, Piscataway, NJ).

Similarly, gram positive bacteria, notably *Bacillus* species and particularly *Bacillus licheniformis*, are used in commercial scale production of heterologous proteins and can be adapted to the expression of APS biosynthetic genes (e.g. Quax *et al.*, *In: Industrial Microorganisms: Basic and Applied Molecular Genetics*, Eds.: Baltz *et al.*, American Society for Microbiology, Washington (1993)). Regulatory signals from a highly expressed *Bacillus* gene (e.g. amylase promoter, Quax *et al.*, *supra*) are used to generate transcriptional fusions with the APS biosynthetic genes.

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In some instances, high level expression of bacterial genes has been achieved using yeast systems, such as the methylotrophic yeast *Pichia pastoris* (Sreekrishna, *In: Industrial microorganisms: basic and applied molecular genetics*, Baltz, Hegeman, and Skatrud eds., American Society for Microbiology, Washington (1993)). The APS gene(s) of interest are positioned behind 5' regulatory sequences of the *Pichia* alcohol oxidase gene in vectors such as pHIL-D1 and pHIL-D2 (Sreekrishna, *supra*). Such vectors are used to transform *Pichia* and introduce the heterologous DNA into the yeast genome. Likewise, the yeast *Saccharomyces cerevisiae* has been used to express heterologous bacterial genes (*e.g.* Dequin & Barre, *Biotechnology* 12:173-177 (1994)). The yeast *Kluyveromyces lactis* is also a suitable host for heterologous gene expression (*e.g.* van den Berg *et al.*, *Biotechnology* 8:135-139 (1990)).

Overexpression of APS genes in organisms such as *E. coli*, *Bacillus* and yeast, which are known for their rapid growth and multiplication, will enable fermentation-production of larger quantities of APSs. The choice of organism may be restricted by the possible susceptibility of the organism to the APS being overproduced; however, the likely susceptibility can be determined by the procedures outlined in Section J. The APSs can be isolated and purified from such cultures (see "G") for use in the control of microorganisms such as fungi and bacteria.

I. Expression of Antibiotic Biosynthetic Genes In Microbial Hosts for Biocontrol Purposes

The cloned APS biosynthetic genes of this invention can be utilized to increase the efficacy of biocontrol strains of various microorganisms. One possibility is the transfer of the genes for a particular APS back into its native host under stronger transcriptional regulation to cause the production of larger quantities of the APS. Another possibility is the transfer of genes to a heterologous host, causing production in the heterologous host of an APS not normally produced by that host.

Microorganisms which are suitable for the heterologous overexpression of APS genes are all microorganisms which are capable of colonizing plants or the rhizosphere. As such they will be brought into contact with phytopathogenic fungi causing an inhibition of their growth. These include gram-negative microorganisms such as *Pseudomonas*, *Enterobacter* and

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Serratia, the gram-positive microorganism *Bacillus* and *Streptomyces spp.* and the fungi *Trichoderma* and *Gliocladium*. Particularly preferred heterologous hosts are *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas cepacia*, *Pseudomonas aureofaciens*, *Pseudomonas aurantiaca*, *Enterobacter cloacae*, *Serratia marcescens*, *Bacillus subtilis*, *Bacillus cereus*, *Trichoderma viride*, *Trichoderma harzianum* and *Gliocladium virens*.

Example 27: Expression of APS Biosynthetic Genes in *E. coli* and Other Gram-Negative Bacteria

Many genes have been expressed in gram-negative bacteria in a heterologous manner. Example 11 describes the expression of genes for pyrrolnitrin biosynthesis in *E. coli* using the expression vector pKK223-3 (Pharmacia catalogue # 27-4935-01). This vector has a strong *tac* promoter (Brosius, J. et al., *Proc. Natl. Acad. Sci. USA* 81) regulated by the *lac* repressor and induced by IPTG. A number of other expression systems have been developed for use in *E. coli* and some are detailed in Examples 14-17 above. The thermoinducible expression vector pP_L (Pharmacia #27-4946-01) uses a tightly regulated bacteriophage λ promoter which allows for high level expression of proteins. The *lac* promoter provides another means of expression but the promoter is not expressed at such high levels as the *tac* promoter. With the addition of broad host range replicons to some of these expression system vectors, production of antifungal compounds in closely related gram negative-bacteria such as *Pseudomonas*, *Enterobacter*, *Serratia* and *Erwinia* is possible. For example, pLRKD211 (Kaiser & Kroos, *Proc. Natl. Acad. Sci. USA* 81: 5816-5820 (1984)) contains the broad host range replicon *ori T* which allows replication in many gram-negative bacteria.

In *E. coli*, induction by IPTG is required for expression of the *tac* (i.e. *trp-lac*) promoter. When this same promoter (e.g. on wide-host range plasmid pLRKD211) is introduced into *Pseudomonas* it is constitutively active without induction by IPTG. This *trp-lac* promoter can be placed in front of any gene or operon of interest for expression in *Pseudomonas* or any other closely related bacterium for the purposes of the constitutive expression of such a gene. If the operon of interest contains the information for the biosynthesis of an APS, then an otherwise biocontrol-minus strain of a gram-negative bacterium may be able to protect plants against a variety of fungal diseases. Thus, genes for antifungal compounds can therefore be placed behind a strong constitutive promoter, transferred to a bacterium that

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normally does not produce antifungal products and which has plant or rhizosphere colonizing properties turning these organisms into effective biocontrol strains. Other possible promoters can be used for the constitutive expression of APS genes in gram-negative bacteria. These include, for example, the promoter from the *Pseudomonas* regulatory genes *gafA* and *lemA* (WO 94/01561) and the *Pseudomonas savastanoi* IAA operon promoter (Gaffney *et al.*, *J. Bacteriol.* 172: 5593-5601 (1990)).

The synthetic Prn operon with the tac promoter as described in example 11a was inserted into two broad host range vectors that replicate in a wide range of Gram negative bacteria. The first vector, pRK290 (Ditta *et al* 1980. PNAS 77(12) pp. 7347-7351), is a low copy number plasmid and the second vector, pBBR1MCS (Kovach *et al* 1994, Biotechniques 16(5):800-802), a medium copy number plasmid. Constructs of both vectors containing the Prn genes were introduced into a number of Gram negative bacterial strains and assayed for production of Pyrrolnitrin by TLC and HPLC. A number of strains were shown to heterologously produce Pyrrolnitrin. These include *E.coli*, *Pseudomonas sp.* (MOCG133, MOCG380, MOCG382, BL897, BL1889, BL2595) and *Enterobacter taylorae* (MOCG206).

Example 28: Expression of APS Biosynthetic Genes in Gram-Positive Bacteria

Heterologous expression of genes encoding APS genes in gram-positive bacteria is another means of producing new biocontrol strains. Expression systems for *Bacillus* and *Streptomyces* are the best characterized. The promoter for the erythromycin resistance gene (*ermR*) from *Streptococcus pneumoniae* has been shown to be active in gram-positive aerobes and anaerobes and also in *E.coli* (Trieu-Cuot *et al.*, Nucl Acids Res 18: 3660 (1990)). A further antibiotic resistance promoter from the thiostreptone gene has been used in *Streptomyces* cloning vectors (Bibb, Mol Gen Genet 199: 26-36 (1985)). The shuttle vector pHT3101 is also appropriate for expression in *Bacillus* (Lereclus, FEMS Microbiol Lett 60: 211-218 (1989)). By expressing an operon (such as the pyrrolnitrin operon) or individual APS encoding genes under control of the *ermR* or other promoters it will be possible to convert soil bacilli into strains able to protect plants against microbial diseases. A significant advantage of this approach is that many gram-positive bacteria produce spores which can be used in formulations that produce biocontrol products with a longer shelf life. *Bacillus* and *Streptomyces* species are aggressive colonizers of soils. In fact both produce secondary metabolites including antibiotics active against a broad range of

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organisms and the addition of heterologous antifungal genes including (including those encoding pyrrolnitrin, soraphen, phenazine or cyclic peptides) to gram-positive bacteria may make these organisms even better biocontrol strains.

Example 29: Expression of APS Biosynthetic Genes in Fungi

Trichoderma harzianum and *Gliocladium virens* have been shown to provide varying levels of biocontrol in the field (US 5,165,928 and US 4,996,157, both to Cornell Research Foundation). The successful use of these biocontrol agents will be greatly enhanced by the development of improved strains by the introduction of genes for APSs. This could be accomplished by a number of ways which are well known in the art. One is protoplast mediated transformation of the fungus by PEG or electroporation-mediated techniques. Alternatively, particle bombardment can be used to transform protoplasts or other fungal cells with the ability to develop into regenerated mature structures. The vector pAN7-1, originally developed for *Aspergillus* transformation and now used widely for fungal transformation (Curragh *et al.*, *Mycol. Res.* 97(3): 313-317 (1992); Tooley *et al.*, *Curr. Genet.* 21: 55-60 (1992); Punt *et al.*, *Gene* 56: 117-124 (1987)) is engineered to contain the pyrrolnitrin operon, or any other genes for APS biosynthesis. This plasmid contains the *E. coli* the hygromycin B resistance gene flanked by the *Aspergillus nidulans* *gpd* promoter and the *trpC* terminator (Punt *et al.*, *Gene* 56: 117-124 (1987)).

J. In Vitro Activity of Anti-phytopathogenic Substances Against Plant Pathogens

Example 30: Bioassay Procedures for the Detection of Antifungal Activity

Inhibition of fungal growth by a potential antifungal agent can be determined in a number of assay formats. Macroscopic methods which are commonly used include the agar diffusion assay (Dhingra & Sinclair, *Basic Plant Pathology Methods*, CRC Press, Boca Raton, FLA (1985)) and assays in liquid media (Broekaert *et al.*, *FEMS Microbiol. Lett.* 69: 55-60.(1990)). Both types of assay are performed with either fungal spores or mycelia as inocula. The maintenance of fungal stocks is in accordance with standard mycological procedures. Spores for bioassay are harvested from a mature plate of a fungus by flushing the surface of the culture with sterile water or buffer. A suspension of mycelia is prepared by placing fungus from a plate in a blender and homogenizing until the colony is dispersed. The homogenate is filtered through several layers of cheesecloth so that larger particles are

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excluded. The suspension which passes through the cheesecloth is washed by centrifugation and replacing the supernatant with fresh buffer. The concentration of the mycelial suspension is adjusted empirically, by testing the suspension in the bioassay to be used.

Agar diffusion assays may be performed by suspending spores or mycelial fragments in a solid test medium, and applying the antifungal agent at a point source, from which it diffuses. This may be done by adding spores or mycelia to melted fungal growth medium, then pouring the mixture into a sterile dish and allowing it to gel. Sterile filters are placed on the surface of the medium, and solutions of antifungal agents are spotted onto the filters. After the liquid has been absorbed by the filter, the plates are incubated at the appropriate temperature, usually for 1-2 days. Growth inhibition is indicated by the presence of zones around filters in which spores have not germinated, or in which mycelia have not grown. The antifungal potency of the agent, denoted as the minimal effective dose, may be quantified by spotting serial dilutions of the agent onto filters, and determining the lowest dose which gives an observable inhibition zone. Another agar diffusion assay can be performed by cutting wells into solidified fungal growth medium and placing solutions of antifungal agents into them. The plate is inoculated at a point equidistant from all the wells, usually at the center of the plate, with either a small aliquot of spore or mycelial suspension or a mycelial plug cut directly from a stock culture plate of the fungus. The plate is incubated for several days until the growing mycelia approach the wells, then it is observed for signs of growth inhibition. Inhibition is indicated by the deformation of the roughly circular form which the fungal colony normally assumes as it grows. Specifically, if the mycelial front appears flattened or even concave relative to the uninhibited sections of the plate, growth inhibition has occurred. A minimal effective concentration may be determined by testing diluted solutions of the agent to find the lowest at which an effect can be detected.

Bioassays in liquid media are conducted using suspensions of spores or mycelia which are incubated in liquid fungal growth media instead of solid media. The fungal inocula, medium, and antifungal agent are mixed in wells of a 96-well microtiter plate, and the growth of the fungus is followed by measuring the turbidity of the culture spectrophotometrically. Increases in turbidity correlate with increases in biomass, and are a measure of fungal

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growth. Growth inhibition is determined by comparing the growth of the fungus in the presence of the antifungal agent with growth in its absence. By testing diluted solutions of antifungal inhibitor, a minimal inhibitory concentration or an EC₅₀ may be determined.

Example 31: Bioassay Procedures for the Detection of Antibacterial Activity

A number of bioassays may be employed to determine the antibacterial activity of an unknown compound. The inhibition of bacterial growth in solid media may be assessed by dispersing an inoculum of the bacterial culture in melted medium and spreading the suspension evenly in the bottom of a sterile Petri dish. After the medium has gelled, sterile filter disks are placed on the surface, and aliquots of the test material are spotted onto them. The plate is incubated overnight at an appropriate temperature, and growth inhibition is observed as an area around a filter in which the bacteria have not grown, or in which the growth is reduced compared to the surrounding areas. Pure compounds may be characterized by the determination of a minimal effective dose, the smallest amount of material which gives a zone of inhibited growth. In liquid media, two other methods may be employed. The growth of a culture may be monitored by measuring the optical density of the culture, in actuality the scattering of incident light. Equal inocula are seeded into equal culture volumes, with one culture containing a known amount of a potential antibacterial agent. After incubation at an appropriate temperature, and with appropriate aeration as required by the bacterium being tested, the optical densities of the cultures are compared. A suitable wavelength for the comparison is 600 nm. The antibacterial agent may be characterized by the determination of a minimal effective dose, the smallest amount of material which produces a reduction in the density of the culture, or by determining an EC₅₀, the concentration at which the growth of the test culture is half that of the control. The bioassays described above do not differentiate between bacteriostatic and bacteriocidal effects. Another assay can be performed which will determine the bacteriocidal activity of the agent. This assay is carried out by incubating the bacteria and the active agent together in liquid medium for an amount of time and under conditions which are sufficient for the agent to exert its effect. After this incubation is completed, the bacteria may be either washed by centrifugation and resuspension, or diluted by the addition of fresh medium. In either case, the concentration of the antibacterial agent is reduced to a point at which it is no longer expected to have significant activity. The bacteria are plated and spread on solid medium and the plates are incubated overnight at an appropriate

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temperature for growth. The number of colonies which arise on the plates are counted, and the number which appeared from the mixture which contained the antibacterial agent is compared with the number which arose from the mixture which contained no antibacterial agent. The reduction in colony-forming units is a measure of the bacteriocidal activity of the agent. The bacteriocidal activity may be quantified as a minimal effective dose, or as an EC₅₀, as described above. Bacteria which are used in assays such as these include species of *Agrobacterium*, *Erwinia*, *Clavibacter*, *Xanthomonas*, and *Pseudomonas*.

Example 32: Antipathogenic Activity Determination of APSs

APSs are assayed using the procedures of examples 30 and 31 above to identify the range of fungi and bacteria against which they are active. The APS can be isolated from the cells and culture medium of the host organism normally producing it, or can alternatively be isolated from a heterologous host which has been engineered to produce the APS. A further possibility is the chemical synthesis of APS compounds of known chemical structure, or derivatives thereof.

Example 33: Antimicrobial Activity Determination of Pyrrolnitrin

a) The anti-phytopathogenic activity of a fluorinated 3-cyano-derivative of pyrrolnitrin (designated CGA173506) was observed against the maize fungal phytopathogens *Diplodia maydis*, *Colletotrichum graminicola*, and *Gibberella zeae-maydis*. Spores of the fungi were harvested and suspended in water. Approximately 1000 spores were inoculated into potato dextrose broth and either CGA173506 or water in a total volume of 100 microliters in the wells of 96-well microtiter plates suitable for a plate reader. The compound CGA173506 was obtained as a 50% wettable powder, and a stock suspension was made up at a concentration of 10 mg/ml in sterile water. This stock suspension was diluted with sterile water to provide the 173506 used in the tests. After the spores, medium, and 173506 were mixed, the turbidity in the wells was measured by reading the absorbance at 600 nm in a plate reader. This reading was taken as the background turbidity, and was subtracted from readings taken at later times. After 46 hours of incubation, the presence of 1 microgram/ml of 173506 was determined to reduce the growth of *Diplodia maydis* by 64%, and after 120 hours, the same concentration of 173506 inhibited the growth of *Colletotrichum graminicola* by 50%. After 40 hours of incubation, the presence of 0.5 microgram/ml of 173506 gave 100% inhibition of *Gibberella zeae-maydis*.

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b) Pyrrolnitrin was tested for its effect on the growth of various maize fungal pathogens and inhibited growth of *Bipolaris maydis*, *Colletotrichum graminicola*, *Diplodia maydis*, *Fusarium moniliforme*, *Gibberella zeae* and *Rhizoctania solani*.

To determine growth

To determine growth inhibition autoclaved filter discs (0.25 inch diameter from Schleicher and Schuell) were placed near the perimeter of PDA (DIFCO) plates. Solutions were pipetted onto these filters. 2.5 micrograms pyrrolnitrin (25 microliter) were placed on one filter disc and 25 microliters 63% ethanol were placed on the other disc. Fungal plugs were taken from stock plates and placed in the center of the PDA plates. Each fungus was inoculated onto one plate. the fungus was allowed to grow and inhibition was scored at appropriate times. Inhibition of the fungi indicated above was visually detected.

K. Expression of Antibiotic Biosynthetic Genes in Transgenic Plants

Example 34: Modification of Coding Sequences and Adjacent Sequences

The cloned APS biosynthetic genes described in this application can be modified for expression in transgenic plant hosts. This is done with the aim of producing extractable quantities of APS from transgenic plants (*i.e.* for similar reasons to those described in Section E above), or alternatively the aim of such expression can be the accumulation of APS in plant tissue for the provision of pathogen protection on host plants. A host plant expressing genes for the biosynthesis of an APS and which produces the APS in its cells will have enhanced resistance to phytopathogen attack and will be thus better equipped to withstand crop losses associated with such attack.

The transgenic expression in plants of genes derived from microbial sources may require the modification of those genes to achieve and optimize their expression in plants. In particular, bacterial ORFs which encode separate enzymes but which are encoded by the same transcript in the native microbe are best expressed in plants on separate transcripts. To achieve this, each microbial ORF is isolated individually and cloned within a cassette which provides a plant promoter sequence at the 5' end of the ORF and a plant transcriptional terminator at the 3' end of the ORF. The isolated ORF sequence preferably includes the initiating ATG codon and the terminating STOP codon but may include

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additional sequence beyond the initiating ATG and the STOP codon. In addition, the ORF may be truncated, but still retain the required activity; for particularly long ORFs, truncated versions which retain activity may be preferable for expression in transgenic organisms. By "plant promoter" and "plant transcriptional terminator" it is intended to mean promoters and transcriptional terminators which operate within plant cells. This includes promoters and transcription terminators which may be derived from non-plant sources such as viruses (an example is the Cauliflower Mosaic Virus).

In some cases, modification to the ORF coding sequences and adjacent sequence will not be required. It is sufficient to isolate a fragment containing the ORF of interest and to insert it downstream of a plant promoter. For example, Gaffney *et al.* (Science 261: 754-756 (1993)) have expressed the *Pseudomonas nahG* gene in transgenic plants under the control of the CaMV 35S promoter and the CaMV *tm1* terminator successfully without modification of the coding sequence and with 56 bp of the *Pseudomonas* gene upstream of the ATG still attached, and 165 bp downstream of the STOP codon still attached to the *nahG* ORF. Preferably as little adjacent microbial sequence should be left attached upstream of the ATG and downstream of the STOP codon. In practice, such construction may depend on the availability of restriction sites.

In other cases, the expression of genes derived from microbial sources may provide problems in expression. These problems have been well characterized in the art and are particularly common with genes derived from certain sources such as *Bacillus*. These problems may apply to the APS biosynthetic genes of this invention and the modification of these genes can be undertaken using techniques now well known in the art. The following problems may be encountered:

(1) Codon Usage. The preferred codon usage in plants differs from the preferred codon usage in certain microorganisms. Comparison of the usage of codons within a cloned microbial ORF to usage in plant genes (and in particular genes from the target plant) will enable an identification of the codons within the ORF which should preferably be changed. Typically plant evolution has tended towards a strong preference of the nucleotides C and G in the third base position of monocotyledons, whereas dicotyledons often use the nucleotides A or T at this position. By modifying a gene to incorporate preferred codon

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usage for a particular target transgenic species, many of the problems described below for GC/AT content and illegitimate splicing will be overcome.

(2) GC/AT Content. Plant genes typically have a GC content of more than 35%. ORF sequences which are rich in A and T nucleotides can cause several problems in plants. Firstly, motifs of ATTTA are believed to cause destabilization of messages and are found at the 3' end of many short-lived mRNAs. Secondly, the occurrence of polyadenylation signals such as AATAAA at inappropriate positions within the message is believed to cause premature truncation of transcription. In addition, monocotyledons may recognize AT-rich sequences as splice sites (see below).

(3) Sequences Adjacent to the Initiating Methionine. Plants differ from microorganisms in that their messages do not possess a defined ribosome binding site. Rather, it is believed that ribosomes attach to the 5' end of the message and scan for the first available ATG at which to start translation. Nevertheless, it is believed that there is a preference for certain nucleotides adjacent to the ATG and that expression of microbial genes can be enhanced by the inclusion of a eukaryotic consensus translation initiator at the ATG. Clontech (1993/1994 catalog, page 210) have suggested the sequence GTCGACCATGGTC (SEQ ID NO:7) as a consensus translation initiator for the expression of the *E. coli uidA* gene in plants. Further, Joshi (NAR 15: 6643-6653 (1987)) has compared many plant sequences adjacent to the ATG and suggests the consensus TAAACAATGGCT (SEQ ID NO:8). In situations where difficulties are encountered in the expression of microbial ORFs in plants, inclusion of one of these sequences at the Initiating ATG may improve translation. In such cases the last three nucleotides of the consensus may not be appropriate for inclusion in the modified sequence due to their modification of the second AA residue. Preferred sequences adjacent to the initiating methionine may differ between different plant species. A survey of 14 maize genes located in the GenBank database provided the following results:

Position Before the Initiating ATG in 14 Maize Genes:

	<u>-10</u>	<u>-9</u>	<u>-8</u>	<u>-7</u>	<u>-6</u>	<u>-5</u>	<u>-4</u>	<u>-3</u>	<u>-2</u>	<u>-1</u>
C	3	8	4	6	2	5	6	0	10	7
T	3	0	3	4	3	2	1	1	1	0
A	2	3	1	4	3	2	3	7	2	3
G	6	3	6	0	6	5	4	6	1	5

This analysis can be done for the desired plant species into which APS genes are being incorporated, and the sequence adjacent to the ATG modified to incorporate the preferred nucleotides.

(4) Removal of Illegitimate Splice Sites. Genes cloned from non-plant sources and not optimized for expression in plants may also contain motifs which may be recognized in plants as 5' or 3' splice sites, and be cleaved, thus generating truncated or deleted messages.

Techniques for the modification of coding sequences and adjacent sequences are well known in the art. In cases where the initial expression of a microbial ORF is low and it is deemed appropriate to make alterations to the sequence as described above, then the construction of synthetic genes can be accomplished according to methods well known in the art. These are, for example, described in the published patent disclosures EP 0 385 962 (to Monsanto), EP 0 359 472 (to Lubrizol) and WO 93/07278 (to Ciba-Geigy). In most cases it is preferable to assay the expression of gene constructions using transient assay protocols (which are well known in the art) prior to their transfer to transgenic plants.

Example 35: Construction of Plant Transformation Vectors

Numerous transformation vectors are available for plant transformation, and the genes of this invention can be used in conjunction with any such vectors. The selection of vector for use will depend upon the preferred transformation technique and the target species for transformation. For certain target species, different antibiotic or herbicide selection markers may be preferred. Selection markers used routinely in transformation include the *nptII* gene

which confers resistance to kanamycin and related antibiotics (Messing & Vierra, Gene 19: 259-268 (1982); Bevan *et al.*, Nature 304:184-187 (1983)), the *bar* gene which confers resistance to the herbicide phosphinothricin (White *et al.*, Nucl Acids Res 18: 1062 (1990), Spencer *et al.* Theor Appl Genet 79: 625-631(1990)), the *hph* gene which confers resistance to the antibiotic hygromycin (Blochinger & Diggelmann, Mol Cell Biol 4: 2929-2931), and the *dhfr* gene, which confers resistance to methotrexate (Bourouis *et al.*, EMBO J. 2(7): 1099-1104 (1983)).

(1) Construction of Vectors Suitable for *Agrobacterium* Transformation

Many vectors are available for transformation using *Agrobacterium tumefaciens*. These typically carry at least one T-DNA border sequence and include vectors such as pBIN19 (Bevan, Nucl. Acids Res. (1984)). Below the construction of two typical vectors is described.

Construction of pCIB200 and pCIB2001

The binary vectors pCIB200 and pCIB2001 are used for the construction of recombinant vectors for use with *Agrobacterium* and was constructed in the following manner. pTJS75kan was created by *NarI* digestion of pTJS75 (Schmidhauser & Helinski, J Bacteriol. 164: 446-455 (1985)) allowing excision of the tetracycline-resistance gene, followed by insertion of an *AccI* fragment from pUC4K carrying an NPTII (Messing & Vierra, Gene 19: 259-268 (1982); Bevan *et al.*, Nature 304: 184-187 (1983); McBride *et al.*, Plant Molecular Biology 14: 266-276 (1990)). *XhoI* linkers were ligated to the *EcoRV* fragment of pCIB7 which contains the left and right T-DNA borders, a plant selectable *nos/nptII* chimeric gene and the pUC polylinker (Rothstein *et al.*, Gene 53: 153-161 (1987)), and the *XhoI*-digested fragment was cloned into *Sall*-digested pTJS75kan to create pCIB200 (see also EP 0 332 104, example 19). pCIB200 contains the following unique polylinker restriction sites: *EcoRI*, *SstI*, *KpnI*, *BglII*, *XbaI*, and *Sall*. pCIB2001 is a derivative of pCIB200 which was created by the insertion into the polylinker of additional restriction sites. Unique restriction sites in the polylinker of pCIB2001 are *EcoRI*, *SstI*, *KpnI*, *BglII*, *XbaI*, *Sall*, *MluI*, *BclI*, *AvrII*, *Apal*, *HpaI*, and *StuI*. pCIB2001, in addition to containing these unique restriction sites also has plant and bacterial kanamycin selection, left and right T-DNA borders for *Agrobacterium*-mediated transformation, the RK2-derived *trfA* function for mobilization between *E. coli* and other

hosts, and the *OnT* and *OriV* functions also from RK2. The pCIB2001 polylinker is suitable for the cloning of plant expression cassettes containing their own regulatory signals.

Construction of pCIB10 and Hygromycin Selection Derivatives thereof

The binary vector pCIB10 contains a gene encoding kanamycin resistance for selection in plants, T-DNA right and left border sequences and incorporates sequences from the wide host-range plasmid pRK252 allowing it to replicate in both *E. coli* and *Agrobacterium*. Its construction is described by Rothstein *et al.* (Gene 53: 153-161 (1987)). Various derivatives of pCIB10 have been constructed which incorporate the gene for hygromycin B phosphotransferase described by Gritz *et al.* (Gene 25: 179-188 (1983)). These derivatives enable selection of transgenic plant cells on hygromycin only (pCIB743), or hygromycin and kanamycin (pCIB715, pCIB717).

(2) Construction of Vectors Suitable for non-*Agrobacterium* Transformation.

Transformation without the use of *Agrobacterium tumefaciens* circumvents the requirement for T-DNA sequences in the chosen transformation vector and consequently vectors lacking these sequences can be utilized in addition to vectors such as the ones described above which contain T-DNA sequences. Transformation techniques which do not rely on *Agrobacterium* include transformation via particle bombardment, protoplast uptake (*e.g.* PEG and electroporation) and microinjection. The choice of vector depends largely on the preferred selection for the species being transformed. Below, the construction of some typical vectors is described.

Construction of pCIB3064

pCIB3064 is a pUC-derived vector suitable for direct gene transfer techniques in combination with selection by the herbicide basta (or phosphinothricin). The plasmid pCIB246 comprises the CaMV 35S promoter in operational fusion to the *E. coli* GUS gene and the CaMV 35S transcriptional terminator and is described in the PCT published application WO 93/07278. The 35S promoter of this vector contains two ATG sequences 5' of the start site. These sites were mutated using standard PCR techniques in such a way as to remove the ATGs and generate the restriction sites *SspI* and *PvuII*. The new restriction sites were 96 and 37 bp away from the unique *Sall* site and 101 and 42 bp away from the actual start site. The resultant derivative of pCIB246 was designated pCIB3025.

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The GUS gene was then excised from pCIB3025 by digestion with *Sall* and *SacI*, the termini rendered blunt and religated to generate plasmid pCIB3060. The plasmid pJIT82 was obtained from the John Innes Centre, Norwich and the a 400 bp *SmaI* fragment containing the *bar* gene from *Streptomyces viridochromogenes* was excised and inserted into the *HpaI* site of pCIB3060 (Thompson *et al.* EMBO J 6: 2519-2523 (1987)). This generated pCIB3064 which comprises the *bar* gene under the control of the CaMV 35S promoter and terminator for herbicide selection, a gene for ampicillin resistance (for selection in *E. coli*) and a polylinker with the unique sites *SphI*, *PstI*, *HindIII*, and *BamHI*. This vector is suitable for the cloning of plant expression cassettes containing their own regulatory signals.

Construction of pSOG19 and pSOG35

pSOG35 is a transformation vector which utilizes the *E. coli* gene dihydrofolate reductase (DHFR) as a selectable marker conferring resistance to methotrexate. PCR was used to amplify the 35S promoter (~800 bp), intron 6 from the maize Adh1 gene (~550 bp) and 18 bp of the GUS untranslated leader sequence from pSOG10. A 250 bp fragment encoding the *E. coli* dihydrofolate reductase type II gene was also amplified by PCR and these two PCR fragments were assembled with a *SacI-PstI* fragment from pBI221 (Clontech) which comprised the pUC19 vector backbone and the nopaline synthase terminator. Assembly of these fragments generated pSOG19 which contains the 35S promoter in fusion with the intron 6 sequence, the GUS leader, the DHFR gene and the nopaline synthase terminator. Replacement of the GUS leader in pSOG19 with the leader sequence from Maize Chlorotic Mottle Virus (MCMV) generated the vector pSOG35. pSOG19 and pSOG35 carry the pUC gene for ampicillin resistance and have *HindIII*, *SphI*, *PstI* and *EcoRI* sites available for the cloning of foreign sequences.

Example 36: Requirements for Construction of Plant Expression Cassettes

Gene sequences intended for expression in transgenic plants are firstly assembled in expression cassettes behind a suitable promoter and upstream of a suitable transcription terminator. These expression cassettes can then be easily transferred to the plant transformation vectors described above in example 2-6.

Promoter Selection

The selection of promoter used in expression cassettes will determine the spatial and temporal expression pattern of the transgene in the transgenic plant. Selected promoters will express transgenes in specific cell types (such as leaf epidermal cells, mesophyll cells, root cortex cells) or in specific tissues or organs (roots, leaves or flowers, for example) and this selection will reflect the desired location of biosynthesis of the APS. Alternatively, the selected promoter may drive expression of the gene under a light-induced or other temporally regulated promoter. A further alternative is that the selected promoter be chemically regulated. This would provide the possibility of inducing the induction of the APS only when desired and caused by treatment with a chemical inducer.

Transcriptional Terminators

A variety of transcriptional terminators are available for use in expression cassettes. These are responsible for the termination of transcription beyond the transgene and its correct polyadenylation. Appropriate transcriptional terminators and those which are known to function in plants and include the CaMV 35S terminator, the *tml* terminator, the nopaline synthase terminator, the pea *rbcS* E9 terminator. These can be used in both monocotyledons and dicotyledons.

Sequences for the Enhancement or Regulation of Expression

Numerous sequences have been found to enhance gene expression from within the transcriptional unit and these sequences can be used in conjunction with the genes of this invention to increase their expression in transgenic plants.

Various intron sequences have been shown to enhance expression, particularly in monocotyledonous cells. For example, the introns of the maize *Adh1* gene have been found to significantly enhance the expression of the wild-type gene under its cognate promoter when introduced into maize cells. Intron 1 was found to be particularly effective and enhanced expression in fusion constructs with the chloramphenicol acetyltransferase gene (Callis *et al.*, *Genes Develop* **1**: 1183-1200 (1987)). In the same experimental system, the intron from the maize *bronze1* gene had a similar effect in enhancing expression (Callis *et al.*, *supra*). Intron sequences have been routinely incorporated into plant transformation vectors, typically within the non-translated leader.

A number of non-translated leader sequences derived from viruses are also known to enhance expression, and these are particularly effective in dicotyledonous cells. Specifically, leader sequences from Tobacco Mosaic Virus (TMV, the "Ω-sequence"), Maize Chlorotic Mottle Virus (MCMV), and Alfalfa Mosaic Virus (AMV) have been shown to be effective in enhancing expression (*e.g.* Gallie *et al.* Nucl. Acids Res. 15: 8693-8711 (1987); Skuzeski *et al.* Plant Molec. Biol. 15: 65-79 (1990))

Targeting of the Gene Product Within the Cell

Various mechanisms for targeting gene products are known to exist in plants and the sequences controlling the functioning of these mechanisms have been characterized in some detail. For example, the targeting of gene products to the chloroplast is controlled by a signal sequence found at the aminoterminal end of various proteins and which is cleaved during chloroplast import yielding the mature protein (*e.g.* Comai *et al.* J. Biol. Chem. 263: 15104-15109 (1988)). These signal sequences can be fused to heterologous gene products to effect the import of heterologous products into the chloroplast (van den Broeck *et al.* Nature 313: 358-363 (1985)). DNA encoding for appropriate signal sequences can be isolated from the 5' end of the cDNAs encoding the RUBISCO protein, the CAB protein, the EPSP synthase enzyme, the GS2 protein and many other proteins which are known to be chloroplast localized.

Other gene products are localized to other organelles such as the mitochondrion and the peroxisome (*e.g.* Unger *et al.* Plant Molec. Biol. 13: 411-418 (1989)). The cDNAs encoding these products can also be manipulated to effect the targeting of heterologous gene products to these organelles. Examples of such sequences are the nuclear-encoded ATPases and specific aspartate amino transferase isoforms for mitochondria. Targeting to cellular protein bodies has been described by Rogers *et al.* (Proc. Natl. Acad. Sci. USA 82: 6512-6516 (1985)).

In addition sequences have been characterized which cause the targeting of gene products to other cell compartments. Aminoterminal sequences are responsible for targeting to the ER, the apoplast, and extracellular secretion from aleurone cells (Koehler & Ho, Plant Cell 2: 769-783 (1990)). Additionally, aminoterminal sequences in conjunction with

carboxyterminal sequences are responsible for vacuolar targeting of gene products (Shinshi *et al.* Plant Molec. Biol. 14: 357-368 (1990)).

By the fusion of the appropriate targeting sequences described above to transgene sequences of interest it is possible to direct the transgene product to any organelle or cell compartment. For chloroplast targeting, for example, the chloroplast signal sequence from the RUBISCO gene, the CAB gene, the EPSP synthase gene, or the GS2 gene is fused in frame to the aminoterminal ATG of the transgene. The signal sequence selected should include the known cleavage site and the fusion constructed should take into account any amino acids after the cleavage site which are required for cleavage. In some cases this requirement may be fulfilled by the addition of a small number of amino acids between the cleavage site and the transgene ATG or alternatively replacement of some amino acids within the transgene sequence. Fusions constructed for chloroplast import can be tested for efficacy of chloroplast uptake by *in vitro* translation of *in vitro* transcribed constructions followed by *in vitro* chloroplast uptake using techniques described by (Bartlett *et al.* In: Edelman *et al.* (Eds.) Methods in Chloroplast Molecular Biology, Elsevier. pp 1081-1091 (1982); Wasmann *et al.* Mol. Gen. Genet. 205: 446-453 (1986)). These construction techniques are well known in the art and are equally applicable to mitochondria and peroxisomes. The choice of targeting which may be required for APS biosynthetic genes will depend on the cellular localization of the precursor required as the starting point for a given pathway. This will usually be cytosolic or chloroplastic, although in some cases be mitochondrial or peroxisomal. The gene products of APS biosynthetic genes will not normally require targeting to the ER, the apoplast or the vacuole.

The above described mechanisms for cellular targeting can be utilized not only in conjunction with their cognate promoters, but also in conjunction with heterologous promoters so as to effect a specific cell targeting goal under the transcriptional regulation of a promoter which has an expression pattern different to that of the promoter from which the targeting signal derives.

Example 37: Examples of Expression Cassette Construction

The present invention encompasses the expression of genes encoding APSs under the regulation of any promoter which is expressible in plants, regardless of the origin of the promoter.

Furthermore, the invention encompasses the use of any plant-expressible promoter in conjunction with any further sequences required or selected for the expression of the APS gene. Such sequences include, but are not restricted to, transcriptional terminators, extraneous sequences to enhance expression (such as introns (*e.g. Adh* intron 1), viral sequences (*e. g.* TMV- Ω)), and sequences intended for the targeting of the gene product to specific organelles and cell compartments.

Constitutive Expression: the CaMV 35S Promoter

Construction of the plasmid pCGN1761 is described in the published patent application EP 0 392 225 (example 23). pCGN1761 contains the "double" 35S promoter and the *tml* transcriptional terminator with a unique *EcoRI* site between the promoter and the terminator and has a pUC-type backbone. A derivative of pCGN1761 was constructed which has a modified polylinker which includes *NotI* and *XhoI* sites in addition to the existing *EcoRI* site. This derivative was designated pCGN1761ENX. pCGN1761ENX is useful for the cloning of cDNA sequences or gene sequences (including microbial ORF sequences) within its polylinker for the purposes of their expression under the control of the 35S promoter in transgenic plants. The entire 35S promoter-gene sequence-*tml* terminator cassette of such a construction can be excised by *HindIII*, *SphI*, *Sall*, and *XbaI* sites 5' to the promoter and *XbaI*, *BamHI* and *BglII* sites 3' to the terminator for transfer to transformation vectors such as those described above in example 35. Furthermore, the double 35S promoter fragment can be removed by 5' excision with *HindIII*, *SphI*, *Sall*, *XbaI*, or *PstI*, and 3' excision with any of the polylinker restriction sites (*EcoRI*, *NotI* or *XhoI*) for replacement with another promoter.

Modification of pCGN1761ENX by Optimization of the Translational Initiation Site

For any of the constructions described in this section, modifications around the cloning sites can be made by the introduction of sequences which may enhance translation. This is particularly useful when genes derived from microorganisms are to be introduced into plant

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expression cassettes as these genes may not contain sequences adjacent to their initiating methionine which may be suitable for the initiation of translation in plants. In cases where genes derived from microorganisms are to be cloned into plant expression cassettes at their ATG it may be useful to modify the site of their insertion to optimize their expression. Modification of pCGN1761ENX is described by way of example to incorporate one of several optimized sequences for plant expression (e.g. Joshi, NAR 15: 6643-6653 (1987)).

pCGN1761ENX is cleaved with *SphI*, treated with T4 DNA polymerase and religated, thus destroying the *SphI* site located 5' to the double 35S promoter. This generates vector pCGN1761ENX/Sph-. pCGN1761ENX/Sph- is cleaved with *EcoRI*, and ligated to an annealed molecular adaptor of the sequence 5'-AATTCTAAAGCATGCCGATCGG-3' (SEQ ID NO:9)/5'-AATTCGATCGGCATGCTTTA-3' (SEQ ID NO:10). This generates the vector pCGNSENX which incorporates the *quasi*-optimized plant translational initiation sequence TAAA-C adjacent to the ATG which is itself part of an *SphI* site which is suitable for cloning heterologous genes at their initiating methionine. Downstream of the *SphI* site, the *EcoRI*, *NotI*, and *XhoI* sites are retained.

An alternative vector is constructed which utilizes an *NcoI* site at the initiating ATG. This vector, designated pCGN1761NENX is made by inserting an annealed molecular adaptor of the sequence 5'-AATTCTAAACCATGGCGATCGG-3' (SEQ ID NO:11) / 5'-AATTCGATCGCCATGGTTTA-3' (SEQ ID NO:12) at the pCGN1761ENX *EcoRI* site (Sequence ID's 14 and 15). Thus, the vector includes the *quasi*-optimized sequence TAAACC adjacent to the initiating ATG which is within the *NcoI* site. Downstream sites are *EcoRI*, *NotI*, and *XhoI*. Prior to this manipulation, however, the two *NcoI* sites in the pCGN1761ENX vector (at upstream positions of the 5' 35S promoter unit) are destroyed using similar techniques to those described above for *SphI* or alternatively using "inside-outside" PCR (Innes *et al.* PCR Protocols: A guide to methods and applications. Academic Press, New York (1990); see Example 41). This manipulation can be assayed for any possible detrimental effect on expression by insertion of any plant cDNA or reporter gene sequence into the cloning site followed by routine expression analysis in plants.

Expression under a Chemically Regulatable Promoter

This section describes the replacement of the double 35S promoter in pCGN1761ENX with any promoter of choice; by way of example the chemically regulated PR-1a promoter is described. The promoter of choice is preferably excised from its source by restriction enzymes, but can alternatively be PCR-amplified using primers which carry appropriate terminal restriction sites. Should PCR-amplification be undertaken, then the promoter should be resequenced to check for amplification errors after the cloning of the amplified promoter in the target vector. The chemically regulatable tobacco PR-1a promoter is cleaved from plasmid pCIB1004 (see EP 0 332 104, example 21 for construction) and transferred to plasmid pCGN1761ENX. pCIB1004 is cleaved with *NcoI* and the resultant 3' overhang of the linearized fragment is rendered blunt by treatment with T4 DNA polymerase. The fragment is then cleaved with *HindIII* and the resultant PR-1a promoter containing fragment is gel purified and cloned into pCGN1761ENX from which the double 35S promoter has been removed. This is done by cleavage with *XhoI* and blunting with T4 polymerase, followed by cleavage with *HindIII* and isolation of the larger vector-terminator containing fragment into which the pCIB1004 promoter fragment is cloned. This generates a pCGN1761ENX derivative with the PR-1a promoter and the *tm1* terminator and an intervening polylinker with unique *EcoRI* and *NotI* sites. Selected APS genes can be inserted into this vector, and the fusion products (*i.e.* promoter-gene-terminator) can subsequently be transferred to any selected transformation vector, including those described in this application.

Constitutive Expression: the Actin Promoter

Several isoforms of actin are known to be expressed in most cell types and consequently the actin promoter is a good choice for a constitutive promoter. In particular, the promoter from the rice *Act1* gene has been cloned and characterized (McElroy *et al.* Plant Cell 2: 163-171 (1990)). A 1.3 kb fragment of the promoter was found to contain all the regulatory elements required for expression in rice protoplasts. Furthermore, numerous expression vectors based on the *Act1* promoter have been constructed specifically for use in monocotyledons (McElroy *et al.* Mol. Gen. Genet. 231: 150-160 (1991)). These incorporate the *Act1*-intron 1, *Adh1* 5' flanking sequence and *Adh1*-intron 1 (from the maize alcohol dehydrogenase gene) and sequence from the CaMV 35S promoter. Vectors showing highest expression were fusions of 35S and the *Act1* intron or the *Act1* 5' flanking sequence

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and the *Act1* intron. Optimization of sequences around the initiating ATG (of the GUS reporter gene) also enhanced expression. The promoter expression cassettes described by McElroy *et al.* (Mol. Gen. Genet. 231: 150-160 (1991)) can be easily modified for the expression of APS biosynthetic genes and are particularly suitable for use in monocotyledonous hosts. For example, promoter containing fragments can be removed from the McElroy constructions and used to replace the double 35S promoter in pCGN1761ENX, which is then available for the insertion of specific gene sequences. The fusion genes thus constructed can then be transferred to appropriate transformation vectors. In a separate report the rice *Act1* promoter with its first intron has also been found to direct high expression in cultured barley cells (Chibbar *et al.* Plant Cell Rep. 12: 506-509 (1993)).

Constitutive Expression: the Ubiquitin Promoter

Ubiquitin is another gene product known to accumulate in many call types and its promoter has been cloned from several species for use in transgenic plants (*e.g.* sunflower - Binet *et al.* Plant Science 79: 87-94 (1991), maize - Christensen *et al.* Plant Molec. Biol. 12: 619-632 (1989)). The maize ubiquitin promoter has been developed in transgenic monocot systems and its sequence and vectors constructed for monocot transformation are disclosed in the patent publication EP 0 342 926 (to Lubrizol). Further, Taylor *et al.* (Plant Cell Rep. 12: 491-495 (1993)) describe a vector (pAHC25) which comprises the maize ubiquitin promoter and first intron and its high activity in cell suspensions of numerous monocotyledons when introduced via microprojectile bombardment. The ubiquitin promoter is clearly suitable for the expression of APS biosynthetic genes in transgenic plants, especially monocotyledons. Suitable vectors are derivatives of pAHC25 or any of the transformation vectors described in this application, modified by the introduction of the appropriate ubiquitin promoter and/or intron sequences.

Root Specific Expression

A preferred pattern of expression for the APSs of the instant invention is root expression. Root expression is particularly useful for the control of soil-borne phytopathogens such as *Rhizoctonia* and *Pythium*. Expression of APSs only in root tissue would have the advantage of controlling root invading phytopathogens, without a concomitant accumulation of APS in leaf and flower tissue and seeds. A suitable root promoter is that described by de

Framond (FEBS 290: 103-106 (1991)) and also in the published patent application EP 0 452 269 (to Ciba-Geigy). This promoter is transferred to a suitable vector such as pCGN1761ENX for the insertion of an APS gene of interest and subsequent transfer of the entire promoter-gene-terminator cassette to a transformation vector of interest.

Wound Inducible Promoters

Wound-inducible promoters are particularly suitable for the expression of APS biosynthetic genes because they are typically active not just on wound induction, but also at the sites of phytopathogen infection. Numerous such promoters have been described (*e.g.* Xu *et al.* Plant Molec. Biol. 22: 573-588 (1993), Logemann *et al.* Plant Cell 1: 151-158 (1989), Rohrmeier & Lehle, Plant Molec. Biol. 22: 783-792 (1993), Firek *et al.* Plant Molec. Biol. 22: 129-142 (1993), Warner *et al.* Plant J. 3: 191-201 (1993)) and all are suitable for use with the instant invention. Logemann *et al.* (*supra*) describe the 5' upstream sequences of the dicotyledonous potato *wun1* gene. Xu *et al.* (*supra*) show that a wound inducible promoter from the dicotyledon potato (*pin2*) is active in the monocotyledon rice. Further, Rohrmeier & Lehle (*supra*) describe the cloning of the maize *Wip1* cDNA which is wound induced and which can be used to isolated the cognate promoter using standard techniques. Similarly, Firek *et al.* (*supra*) and Warner *et al.* (*supra*) have described a wound induced gene from the monocotyledon *Asparagus officinalis* which is expressed at local wound and pathogen invasion sites. Using cloning techniques well known in the art, these promoters can be transferred to suitable vectors, fused to the APS biosynthetic genes of this invention, and used to express these genes at the sites of phytopathogen infection.

Pith Preferred Expression

Patent Application WO 93/07278 (to Ciba-Geigy) describes the isolation of the maize *tpA* gene which is preferentially expressed in pith cells. The gene sequence and promoter extending up to nucleotide -1726 from the start of transcription are presented. Using standard molecular biological techniques, this promoter or parts thereof, can be transferred to a vector such as pCGN1761 where it can replace the 35S promoter and be used to drive the expression of a foreign gene in a pith-preferred manner. In fact fragments containing the pith-preferred promoter or parts thereof can be transferred to any vector and modified for utility in transgenic plants.

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Pollen-Specific Expression

Patent Application WO 93/07278 (to Ciba-Geigy) further describes the isolation of the maize calcium-dependent protein kinase (CDPK) gene which is expressed in pollen cells. The gene sequence and promoter extend up to 1400 bp from the start of transcription. Using standard molecular biological techniques, this promoter or parts thereof, can be transferred to a vector such as pCGN1761 where it can replace the 35S promoter and be used to drive the expression of a foreign gene in a pollen-specific manner. In fact fragments containing the pollen-specific promoter or parts thereof can be transferred to any vector and modified for utility in transgenic plants.

Leaf-Specific Expression

A maize gene encoding phosphoenol carboxylase (PEPC) has been described by Hudspeth & Grula (Plant Molec Biol 12: 579-589 (1989)). Using standard molecular biological techniques the promoter for this gene can be used to drive the expression of any gene in a leaf-specific manner in transgenic plants.

Expression with Chloroplast Targeting

Chen & Jagendorf (J. Biol. Chem. 268: 2363-2367 (1993) have described the successful use of a chloroplast transit peptide for import of a heterologous transgene. This peptide used is the transit peptide from the *rbcs* gene from *Nicotiana plumbaginifolia* (Poulsen *et al.* Mol. Gen. Genet. 205: 193-200 (1986)). Using the restriction enzymes *DraI* and *SphI*, or *Tsp509I* and *SphI* the DNA sequence encoding this transit peptide can be excised from plasmid prbcS-8B (Poulsen *et al. supra*) and manipulated for use with any of the constructions described above. The *DraI-SphI* fragment extends from -58 relative to the initiating *rbcs* ATG to, and including, the first amino acid (also a methionine) of the mature peptide immediately after the import cleavage site, whereas the *Tsp509I-SphI* fragment extends from -8 relative to the initiating *rbcs* ATG to, and including, the first amino acid of the mature peptide. Thus, these fragment can be appropriately inserted into the polylinker of any chosen expression cassette generating a transcriptional fusion to the untranslated leader of the chosen promoter (e.g. 35S, PR-1a, actin, ubiquitin *etc.*), whilst enabling the insertion of a required APS gene in correct fusion downstream of the transit peptide. Constructions of this kind are routine in the art. For example, whereas the *DraI* end is already blunt, the 5' *Tsp509I* site may be rendered blunt by T4 polymerase treatment, or

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may alternatively be ligated to a linker or adaptor sequence to facilitate its fusion to the chosen promoter. The 3' *SphI* site may be maintained as such, or may alternatively be ligated to adaptor or linker sequences to facilitate its insertion into the chosen vector in such a way as to make available appropriate restriction sites for the subsequent insertion of a selected APS gene. Ideally the ATG of the *SphI* site is maintained and comprises the first ATG of the selected APS gene. Chen & Jagendorf (*supra*) provide consensus sequences for ideal cleavage for chloroplast import, and in each case a methionine is preferred at the first position of the mature protein. At subsequent positions there is more variation and the amino acid may not be so critical. In any case, fusion constructions can be assessed for efficiency of import *in vitro* using the methods described by Bartlett *et al.* (In: Edelman *et al.* (Eds.) *Methods in Chloroplast Molecular Biology*, Elsevier. pp 1081-1091 (1982)) and Wasmann *et al.* (*Mol. Gen. Genet.* 205: 446-453 (1986)). Typically the best approach may be to generate fusions using the selected APS gene with no modifications at the aminoterminalus, and only to incorporate modifications when it is apparent that such fusions are not chloroplast imported at high efficiency, in which case modifications may be made in accordance with the established literature (Chen & Jagendorf, *supra*; Wasman *et al.*, *supra*; Ko & Ko, *J. Biol. Chem.* 267: 13910-13916 (1992)).

A preferred vector is constructed by transferring the *DraI-SphI* transit peptide encoding fragment from *prbcS-8B* to the cloning vector *pCGN1761ENX/Sph-*. This plasmid is cleaved with *EcoRI* and the termini rendered blunt by treatment with T4 DNA polymerase. Plasmid *prbcS-8B* is cleaved with *SphI* and ligated to an annealed molecular adaptor of the sequence 5'-CCAGCTGGAATTCCG-3' (SEQ ID NO:13)/5'-CGGAATTCAGCTGGCATG-3' (SEQ ID NO:14). The resultant product is 5'-terminally phosphorylated by treatment with T4 kinase. Subsequent cleavage with *DraI* releases the transit peptide encoding fragment which is ligated into the blunt-end ex-*EcoRI* sites of the modified vector described above. Clones oriented with the 5' end of the insert adjacent to the 3' end of the 35S promoter are identified by sequencing. These clones carry a DNA fusion of the 35S leader sequence to the *rbcS-8A* promoter-transit peptide sequence extending from -58 relative to the *rbcS* ATG to the ATG of the mature protein, and including at that position a unique *SphI* site, and a newly created *EcoRI* site, as well as the existing *NotI* and *XhoI* sites of *pCGN1761ENX*. This new vector is designated *pCGN1761/CT*. DNA sequences are transferred to *pCGN1761/CT* in frame by amplification using PCR techniques and incorporation of an

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SphI, *NspHI*, or *NlaIII* site at the amplified ATG, which following restriction enzyme cleavage with the appropriate enzyme is ligated into *SphI*-cleaved pCGN1761/CT. To facilitate construction, it may be required to change the second amino acid of the cloned gene, however, in almost all cases the use of PCR together with standard site directed mutagenesis will enable the construction of any desired sequence around the cleavage site and first methionine of the mature protein.

A further preferred vector is constructed by replacing the double 35S promoter of pCGN1761ENX with the *BamHI-SphI* fragment of *prbcS-8A* which contains the full-length light regulated *rbcs-8A* promoter from nucleotide -1038 (relative to the transcriptional start site) up to the first methionine of the mature protein. The modified pCGN1761 with the destroyed *SphI* site is cleaved with *PstI* and *EcoRI* and treated with T4 DNA polymerase to render termini blunt. *prbcS-8A* is cleaved *SphI* and ligated to the annealed molecular adaptor of the sequence described above. The resultant product is 5'-terminally phosphorylated by treatment with T4 kinase. Subsequent cleavage with *BamHI* releases the promoter-transit peptide containing fragment which is treated with T4 DNA polymerase to render the *BamHI* terminus blunt. The promoter-transit peptide fragment thus generated is cloned into the prepared pCGN1761ENX vector, generating a construction comprising the *rbcs-8A* promoter and transit peptide with an *SphI* site located at the cleavage site for insertion of heterologous genes. Further, downstream of the *SphI* site there are *EcoRI* (re-created), *NotI*, and *XhoI* cloning sites. This construction is designated pCGN1761*rbcs*/CT.

Similar manipulations can be undertaken to utilize other GS2 chloroplast transit peptide encoding sequences from other sources (monocotyledonous and dicotyledonous) and from other genes. In addition, similar procedures can be followed to achieve targeting to other subcellular compartments such as mitochondria.

Example 38: Techniques for the Isolation of New Promoters Suitable for the Expression of APS Genes

New promoters are isolated using standard molecular biological techniques including any of the techniques described below. Once isolated, they are fused to reporter genes such as GUS or LUC and their expression pattern in transgenic plants analyzed (Jefferson *et al.*

EMBO J. 6: 3901-3907 (1987); Ow *et al.* Science 234: 856-859 (1986)). Promoters which show the desired expression pattern are fused to APS genes for expression *in planta*.

Subtractive cDNA Cloning

Subtractive cDNA cloning techniques are useful for the generation of cDNA libraries enriched for a particular population of mRNAs (*e.g.* Hara *et al.* Nucl. Acids Res. 19: 1097-7104 (1991)). Recently, techniques have been described which allow the construction of subtractive libraries from small amounts of tissue (Sharma *et al.* Biotechniques 15: 610-612 (1993)). These techniques are suitable for the enrichment of messages specific for tissues which may be available only in small amounts such as the tissue immediately adjacent to wound or pathogen infection sites.

Differential Screening by Standard Plus/Minus Techniques

λ phage carrying cDNAs derived from different RNA populations (*viz.* root versus whole plant, stem specific versus whole plant, local pathogen infection points versus whole plant, *etc.*) are plated at low density and transferred to two sets of hybridization filters (for a review of differential screening techniques see Calvet, *Pediatr. Nephrol.* 5: 751-757 (1991)). cDNAs derived from the "choice" RNA population are hybridized to the first set and cDNAs from whole plant RNA are hybridized to the second set of filters. Plaques which hybridize to the first probe, but not to the second, are selected for further evaluation. They are picked and their cDNA used to screen Northern blots of "choice" RNA versus RNA from various other tissues and sources. Clones showing the required expression pattern are used to clone gene sequences from a genomic library to enable the isolation of the cognate promoter. Between 500 and 5000 bp of the cloned promoter is then fused to a reporter gene (*e.g.* GUS, LUC) and reintroduced into transgenic plants for expression analysis.

Differential Screening by Differential Display

RNA is isolated from different sources *i.e.* the choice source and whole plants as control, and subjected to the differential display technique of Liang and Pardee (Science 257: 967-971 (1992)). Amplified fragments which appear in the choice RNA, but not the control are gel purified and used as probes on Northern blots carrying different RNA samples as described above. Fragments which hybridize selectively to the required RNA are cloned and used as probes to isolate the cDNA and also a genomic DNA fragment from which the

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promoter can be isolated. The isolated promoter is fused to a GUS or LUC reporter gene as described above to assess its expression pattern in transgenic plants.

Promoter Isolation Using "Promoter Trap" Technology

The insertion of promoterless reporter genes into transgenic plants can be used to identify sequences in a host plant which drive expression in desired cell types or with a desired strength. Variations of this technique is described by Ott & Chua (Mol. Gen. Genet. 223: 169-179 (1990)) and Kertbundit *et al.* (Proc. Natl. Acad. Sci. USA 88: 5212-5216 (1991)). In standard transgenic experiments the same principle can be extended to identify enhancer elements in the host genome where a particular transgene may be expressed at particularly high levels.

Example 39: Transformation of Dicotyledons

Transformation techniques for dicotyledons are well known in the art and include *Agrobacterium*-based techniques and techniques which do not require *Agrobacterium*. Non-*Agrobacterium* techniques involve the uptake of exogenous genetic material directly by protoplasts or cells. This can be accomplished by PEG or electroporation mediated uptake, particle bombardment-mediated delivery, or microinjection. Examples of these techniques are described by Paszkowski *et al.*, EMBO J 3: 2717-2722 (1984), Potrykus *et al.*, Mol. Gen. Genet. 199: 169-177 (1985), Reich *et al.*, Biotechnology 4: 1001-1004 (1986), and Klein *et al.*, Nature 327: 70-73 (1987). In each case the transformed cells are regenerated to whole plants using standard techniques known in the art.

Agrobacterium-mediated transformation is a preferred technique for transformation of dicotyledons because of its high efficiency of transformation and its broad utility with many different species. The many crop species which are routinely transformable by *Agrobacterium* include tobacco, tomato, sunflower, cotton, oilseed rape, potato, soybean, alfalfa and poplar (EP 0 317 511 (cotton), EP 0 249 432 (tomato, to Calgene), WO 87/07299 (*Brassica*, to Calgene), US 4,795,855 (poplar)). *Agrobacterium* transformation typically involves the transfer of the binary vector carrying the foreign DNA of interest (*e.g.* pCIB200 or pCIB2001) to an appropriate *Agrobacterium* strain which may depend of the complement of *vir* genes carried by the host *Agrobacterium* strain either on a co-resident Ti plasmid or chromosomally (*e.g.* strain CIB542 for pCIB200 and pCIB2001 (Uknes *et al.*

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Plant Cell 5: 159-169 (1993)). The transfer of the recombinant binary vector to *Agrobacterium* is accomplished by a triparental mating procedure using *E. coli* carrying the recombinant binary vector, a helper *E. coli* strain which carries a plasmid such as pRK2013 and which is able to mobilize the recombinant binary vector to the target *Agrobacterium* strain. Alternatively, the recombinant binary vector can be transferred to *Agrobacterium* by DNA transformation (Höfgen & Willmitzer, Nucl. Acids Res. 16: 9877(1988)).

Transformation of the target plant species by recombinant *Agrobacterium* usually involves co-cultivation of the *Agrobacterium* with explants from the plant and follows protocols well known in the art. Transformed tissue is regenerated on selectable medium carrying the antibiotic or herbicide resistance marker present between the binary plasmid T-DNA borders.

Example 40: Transformation of Monocotyledons

Transformation of most monocotyledon species has now also become routine. Preferred techniques include direct gene transfer into protoplasts using PEG or electroporation techniques, and particle bombardment into callus tissue. Transformations can be undertaken with a single DNA species or multiple DNA species (*i.e.* co-transformation) and both these techniques are suitable for use with this invention. Co-transformation may have the advantage of avoiding complex vector construction and of generating transgenic plants with unlinked loci for the gene of interest and the selectable marker, enabling the removal of the selectable marker in subsequent generations, should this be regarded desirable. However, a disadvantage of the use of co-transformation is the less than 100% frequency with which separate DNA species are integrated into the genome (Schocher *et al.* Biotechnology 4: 1093-1096 (1986)).

Patent Applications EP 0 292 435 (to Ciba-Geigy), EP 0 392 225 (to Ciba-Geigy) and WO 93/07278 (to Ciba-Geigy) describe techniques for the preparation of callus and protoplasts from an elite inbred line of maize, transformation of protoplasts using PEG or electroporation, and the regeneration of maize plants from transformed protoplasts. Gordon-Kamm *et al.* (Plant Cell 2: 603-618 (1990)) and Fromm *et al.* (Biotechnology 8: 833-839 (1990)) have published techniques for transformation of A188-derived maize line using particle bombardment. Furthermore, application WO 93/07278 (to Ciba-Geigy) and Koziel

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et al. (Biotechnology 11: 194-200 (1993)) describe techniques for the transformation of elite inbred lines of maize by particle bombardment. This technique utilizes immature maize embryos of 1.5-2.5 mm length excised from a maize ear 14-15 days after pollination and a PDS-1000He Biolistics device for bombardment.

Transformation of rice can also be undertaken by direct gene transfer techniques utilizing protoplasts or particle bombardment. Protoplast-mediated transformation has been described for *Japonica*-types and *Indica*-types (Zhang *et al.*, Plant Cell Rep 7: 379-384 (1988); Shimamoto *et al.* Nature 338: 274-277 (1989); Datta *et al.* Biotechnology 8: 736-740 (1990)). Both types are also routinely transformable using particle bombardment (Christou *et al.* Biotechnology 9: 957-962 (1991)).

Patent Application EP 0 332 581 (to Ciba-Geigy) describes techniques for the generation, transformation and regeneration of Pooidae protoplasts. These techniques allow the transformation of *Dactylis* and wheat. Furthermore, wheat transformation was been described by Vasil *et al.* (Biotechnology 10: 667-674 (1992)) using particle bombardment into cells of type C long-term regenerable callus, and also by Vasil *et al.* (Biotechnology 11: 1553-1558 (1993)) and Weeks *et al.* (Plant Physiol. 102: 1077-1084 (1993)) using particle bombardment of immature embryos and immature embryo-derived callus. A preferred technique for wheat transformation, however, involves the transformation of wheat by particle bombardment of immature embryos and includes either a high sucrose or a high maltose step prior to gene delivery. Prior to bombardment, any number of embryos (0.75-1 mm in length) are plated onto MS medium with 3% sucrose (Murashiga & Skoog, Physiologia Plantarum 15: 473-497 (1962)) and 3 mg/l 2,4-D for induction of somatic embryos which is allowed to proceed in the dark. On the chosen day of bombardment, embryos are removed from the induction medium and placed onto the osmoticum (*i.e.* induction medium with sucrose or maltose added at the desired concentration, typically 15%). The embryos are allowed to plasmolyze for 2-3 h and are then bombarded. Twenty embryos per target plate is typical, although not critical. An appropriate gene-carrying plasmid (such as pCIB3064 or pSG35) is precipitated onto micrometer size gold particles using standard procedures. Each plate of embryos is shot with the DuPont Biolistics® helium device using a burst pressure of ~1000 psi using a standard 80 mesh screen. After bombardment, the embryos are placed back into the dark to recover for about 24 h (still on

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osmoticum). After 24 hrs, the embryos are removed from the osmoticum and placed back onto induction medium where they stay for about a month before regeneration. Approximately one month later the embryo explants with developing embryogenic callus are transferred to regeneration medium (MS + 1 mg/liter NAA, 5 mg/liter GA), further containing the appropriate selection agent (10 mg/l basta in the case of pCIB3064 and 2 mg/l methotrexate in the case of pSOG35). After approximately one month, developed shoots are transferred to larger sterile containers known as "GA7s" which contained half-strength MS, 2% sucrose, and the same concentration of selection agent. Patent application WO 94/13822 describes methods for wheat transformation and is hereby incorporated by reference.

Example 41: Expression of Pyrrolnitrin in Transgenic Plants

The GC content of all four pyrrolnitrin ORFs is between 62 and 68% and consequently no AT-content related problems are anticipated with their expression in plants. It may, however, be advantageous to modify the genes to include codons preferred in the appropriate target plant species. Fusions of the kind described below can be made to any desired promoter with or without modification (*e.g.* for optimized translational initiation in plants or for enhanced expression).

Expression behind the 35S Promoter

Each of the four pyrrolnitrin ORFs is transferred to pBluescript KS II for further manipulation. This is done by PCR amplification using primers homologous to each end of each gene and which additionally include a restriction site to facilitate the transfer of the amplified fragments to the pBluescript vector. For ORF1, the aminoterminal primer includes a *Sall* site and the carboxyterminal primer a *NotI* site. Similarly for ORF2, the aminoterminal primer includes a *Sall* site and the carboxyterminal primer a *NotI* site. For ORF3, the aminoterminal primer includes a *NotI* site and the carboxyterminal primer an *XhoI* site. Similarly for ORF4, the aminoterminal primer includes a *NotI* site and the carboxyterminal primer an *XhoI* site. Thus, the amplified fragments are cleaved with the appropriate restriction enzymes (chosen because they do not cleave within the ORF) and are then ligated into pBluescript, also correspondingly cleaved. The cloning of the individual ORFs in pBluescript facilitates their subsequent manipulation.

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Destruction of internal restriction sites which are required for further construction is undertaken using the procedure of "inside-outside PCR" (Innes *et al.* PCR Protocols: A guide to methods and applications. Academic Press, New York (1990)). Unique restriction sites sought at either side of the site to be destroyed (ideally between 100 and 500 bp from the site to be destroyed) and two separate amplifications are set up. One extends from the unique site left of the site to be destroyed and amplifies DNA up to the site to be destroyed with an amplifying oligonucleotide which spans this site and incorporates an appropriate base change. The second amplification extends from the site to be destroyed up to the unique site rightwards of the site to be destroyed. The oligonucleotide spanning the site to be destroyed in this second reaction incorporates the same base change as in the first amplification and ideally shares an overlap of between 10 and 25 nucleotides with the oligonucleotide from the first reaction. Thus the products of both reactions share an overlap which incorporates the same base change in the restriction site corresponding to that made in each amplification. Following the two amplifications, the amplified products are gel purified (to remove the four oligonucleotide primers used), mixed together and reamplified in a PCR reaction using the two primers spanning the unique restriction sites. In this final PCR reaction the overlap between the two amplified fragments provides the priming necessary for the first round of synthesis. The product of this reactions extends from the leftwards unique restriction site to the rightwards unique restriction site and includes the modified restriction site located internally. This product can be cleaved with the unique sites and inserted into the unmodified gene at the appropriate location by replacing the wild-type fragment.

To render ORF1 free of the first of its two internal *SphI* sites oligonucleotides spanning and homologous to the unique *XmaI* and *EspI* are designed. The *XmaI* oligonucleotide is used in a PCR reaction together with an oligonucleotide spanning the first *SphI* site and which comprises the sequenceCCCCCICATGC.... (lower strand, SEQ ID NO:15), thus introducing a base change into to *SphI* site. A second PCR reaction utilizes an oligonucleotide spanning the *SphI* site (upper strand) comprising the sequenceGCATGAGGGGG.... (SEQ ID NO:16) and is used in combination with the *EspI* site-spanning oligonucleotide. The two products are gel purified and themselves amplified with the *XmaI* and *EspI*-spanning oligonucleotides and the resultant fragment is cleaved with *XmaI* and *EspI* and used to replace the native fragment in the ORF1 clone. According to

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the above description, the modified *SphI* site is GCATGA and does not cause a codon change. Other changes in this site are possible (*i.e.* changing the second nucleotide to a G, T, or A) without corrupting amino acid integrity.

A similar strategy is used to destroy the second *SphI* site in ORF1. In this case, *EspI* is a suitable leftwards-located restriction site, and the rightwards-located restriction site is *PstI*, located close to the 3' end of the gene or alternatively *SstI* which is not found in the ORF sequence, but immediately adjacent in the pBluescript polylinker. In this case an appropriate oligonucleotide is one which spans this site, or alternatively one of the available pBluescript sequencing primers. This *SphI* site is modified to GAATGC or GCATGT or GAATGT. Each of these changes destroys the site without causing a codon change.

To render ORF2 free of its single *SphI* site a similar procedure is used. Leftward restriction sites are provided by *PstI* or *MluI*, and a suitable rightwards restriction site is provided by *SstI* in the pBluescript polylinker. In this case the site is changed to GCTTGC, GCATGC or GCTTGT; these changes maintain amino acid integrity.

ORF3 has no internal *SphI* sites.

In the case of ORF4, *PstI* provides a suitable rightwards unique site, but there is no suitable site located leftwards of the single *SphI* site to be changed. In this case a restriction site in the pBluescript polylinker can be used to the same effect as already described above. The *SphI* site is modified to GGATGC, GTATGC, GAATGC, or GCATGT *etc.*

The removal of *SphI* sites from the pyrrolnitrin biosynthetic genes as described above facilitates their transfer to the pCGN1761SENX vector by amplification using an aminoterminal oligonucleotide primer which incorporates an *SphI* site at the ATG and a carboxyterminal primer which incorporates a restriction site not found in the gene being amplified. The resultant amplified fragment is cleaved with *SphI* and the restriction enzyme cutting the carboxyterminal sequence and cloned into pCGN1761SENX. Suitable restriction enzyme sites for incorporation into the carboxyterminal primer are *NotI* (for all four ORFs), *XhoI* (for ORF3 and ORF4), and *EcoRI* (for ORF4). Given the requirement for the nucleotide C at position 6 within the *SphI* recognition site, in some cases the second codon of the ORF may require changing so as to start with the nucleotide C. This construction

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fuses each ORF at its ATG to the *SphI* sites of the translation-optimized vector pCGN1761SENX in operable linkage to the double 35S promoter. After construction is complete the final gene insertions and fusion points are resequenced to ensure that no undesired base changes have occurred.

By utilizing an aminoterminal oligonucleotide primer which incorporates an *NcoI* site at its ATG instead of an *SphI* site, ORFs 1-4 can also be easily cloned into the translation-optimized vector pCGN1761NENX. None of the four pyrrolnitrin biosynthetic gene ORFs carry an *NcoI* site and consequently there is no requirement in this case to destroy internal restriction sites. Primers for the carboxyterminus of the gene are designed as described above and the cloning is undertaken in a similar fashion. Given the requirement for the nucleotide G at position 6 within the *NcoI* recognition site, in some cases the second codon of the ORF may require changing so as to start with the nucleotide G. This construction fuses each ORF at its ATG to the *NcoI* site of pCGN1761NENX in operable linkage to the double 35S promoter.

The expression cassettes of the appropriate pCGN1761-derivative vectors are transferred to transformation vectors. Where possible multiple expression cassettes are transferred to a single transformation vector so as to reduce the number of plant transformations and crosses between transformants which may be required to produce plants expressing all four ORFs and thus producing pyrrolnitrin.

Expression behind 35S with Chloroplast Targeting

The pyrrolnitrin ORFs 1-4 amplified using oligonucleotides carrying an *SphI* site at their aminoterminal are cloned into the 35S-chloroplast targeted vector pCGN1761/CT. The fusions are made to the *SphI* site located at the cleavage site of the *rbcS* transit peptide. The expression cassettes thus created are transferred to appropriate transformation vectors (see above) and used to generate transgenic plants. As tryptophan, the precursor for pyrrolnitrin biosynthesis, is synthesized in the chloroplast, it may be advantageous to express the biosynthetic genes for pyrrolnitrin in the chloroplast to ensure a ready supply of substrate. Transgenic plants expressing all four ORFs will target all four gene products to the chloroplast and will thus synthesize pyrrolnitrin in the chloroplast.

Expression behind *rbcS* with Chloroplast Targeting

The pyrrolnitrin ORFs 1-4 amplified using oligonucleotides carrying an *SphI* site at their aminoterminal are cloned into the *rbcS*-chloroplast targeted vector pCGN1761*rbcS*/CT. The fusions are made to the *SphI* site located at the cleavage site of the *rbcS* transit peptide. The expression cassettes thus created are transferred to appropriate transformation vectors (see above) and used to generate transgenic plants. As tryptophan, the precursor for pyrrolnitrin biosynthesis, is synthesized in the chloroplast, it may be advantageous to express the biosynthetic genes for pyrrolnitrin in the chloroplast to ensure a ready supply of substrate. Transgenic plants expressing all four ORFs will target all four gene products to the chloroplast and will thus synthesize pyrrolnitrin in the chloroplast. The expression of the four ORFs will, however, be light induced.

Example 42: Expression of Soraphen in Transgenic Plants

Clone p98/1 contains the entirety of the soraphen biosynthetic gene ORF1 which encodes five biosynthetic modules for soraphen biosynthesis. The partially sequenced ORF2 contains the remaining three modules, and further required for soraphen biosynthesis is the soraphen methylase located on the same operon.

Soraphen ORF1 is manipulated for expression in transgenic plants in the following manner. A DNA fragment is amplified from the aminoterminal of ORF1 using PCR and p98/1 as template. The 5' oligonucleotide primer includes either an *SphI* site or an *NcoI* site at the ATG for cloning into the vectors pCGN1761SENX or pCGNNENX respectively. Further, the 5' oligonucleotide includes either the base C (for *SphI* cloning) or the base G (for *NcoI* cloning) immediately after the ATG, and thus the second amino acid of the protein is changed either to a histidine or an aspartate (other amino acids can be selected for position 2 by additionally changing other bases of the second codon). The 3' oligonucleotide for the amplification is located at the first *BglII* site of the ORF and incorporates a distal *EcoRI* site enabling the amplified fragment to be cleaved with *SphI* (or *NcoI*) and *EcoRI*, and then cloned into pCGN1761SENX (or pCGN1761NENX). To facilitate cleavage of the amplified fragments, each oligonucleotide includes several additional bases at its 5' end. The oligonucleotides preferably have 12-30 bp homology to the ORF1 template, in addition to the required restriction sites and additional sequences. This manipulation fuses the aminoterminal ~112 amino acids of ORF1 at its ATG to the *SphI* or *NcoI* sites of the

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translation optimized vectors pCGN1761SENX or pCGN1761NENX in linkage to the double 35S promoter. The remainder of ORF1 is carried on three *Bgl*III fragments which can be sequentially cloned into the unique *Bgl*III site of the above-detailed constructions. The introduction of the first of these fragments is no problem, and requires only the cleavage of the aminoterminal construction with *Bgl*III followed by introduction of the first of these fragments. For the introduction of the two remaining fragments, partial digestion of the aminoterminal construction is required (since this construction now has an additional *Bgl*III site), followed by introduction of the next *Bgl*III fragment. Thus, it is possible to construct a vector containing the entire ~25 kb of soraphen ORF1 in operable fusion to the 35S promoter.

An alternative approach to constructing the soraphen ORF1 by the fusion of sequential restriction fragments is to amplify the entire ORF using PCR. Barnes (Proc. Natl. Acad. Sci USA 91: 2216-2220 (1994)) has recently described techniques for the high-fidelity amplification of fragments by PCR of up to 35 kb, and these techniques can be applied to ORF1. Oligonucleotides specific for each end of ORF1, with appropriate restriction sites added are used to amplify the entire coding region, which is then cloned into appropriate sites in a suitable vector such as pCGN1761 or its derivatives. Typically after PCR amplification, resequencing is advised to ensure that no base changes have arisen in the amplified sequence. Alternatively, a functional assay can be done directly in transgenic plants.

Yet another approach to the expression of the genes for polyketide biosynthesis (such as soraphen) in transgenic plants is the construction, for expression in plants, of transcriptional units which comprise less than the usual complement of modules, and to provide the remaining modules on other transcriptional units. As it is believed that the biosynthesis of polyketide antibiotics such as soraphen is a process which requires the sequential activity of specific modules and that for the synthesis of a specific molecule these activities should be provided in a specific sequence, it is likely that the expression of different transgenes in a plant carrying different modules may lead to the biosynthesis of novel polyketide molecules because the sequential enzymatic nature of the wild-type genes is determined by their configuration on a single molecule. It is assumed that the localization of five specific modules for soraphen biosynthesis on ORF1 is determinatory in the biosynthesis of

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soraphen, and that the expression of, say three modules on one transgene and the other two on another, together with ORF2, may result in biosynthesis of a polyketide with a different molecular structure and possibly with a different antipathogenic activity. This invention encompasses all such deviations of module expression which may result in the synthesis in transgenic organisms of novel polyketides.

Although specific construction details are only provided for ORF1 above, similar techniques are used to express ORF2 and the soraphen methylase in transgenic plants. For the expression of functional soraphen in plants it is anticipated that all three genes must be expressed and this is done as detailed in this specification.

Fusions of the kind described above can be made to any desired promoter with or without modification (*e.g.* for optimized translational initiation in plants or for enhanced expression). As the ORFs identified for soraphen biosynthesis are around 70% GC rich it is not anticipated that the coding sequences should require modification to increase GC content for optimal expression in plants. It may, however, be advantageous to modify the genes to include codons preferred in the appropriate target plant species.

Example 43: Expression of Phenazine in Transgenic Plants

The GC content of all the cloned genes encoding biosynthetic enzymes for phenazine synthesis is between 58 and 65% and consequently no AT-content related problems are anticipated with their expression in plants (although it may be advantageous to modify the genes to include codons preferred in the appropriate target plant species.). Fusions of the kind described below can be made to any desired promoter with or without modification (*e.g.* for optimized translational initiation in plants or for enhanced expression).

Expression behind the 35S Promoter

Each of the three phenazine ORFs is transferred to pBluescript SK II for further manipulation. The *phzB* ORF is transferred as an *EcoRI-BglII* fragment cloned from plasmid pLSP18-6H3del3 containing the entire phenazine operon. This fragment is transferred to the *EcoRI-BamHI* sites of pBluescript SK II. The *phzC* ORF is transferred from pLSP18-6H3del3 as an *XhoI-SmaI* fragment cloned into the *XhoI-SmaI* sites of

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pBluescript II SK. The *phzD* ORF is transferred from pLSP18-6H3del3 as a *BglII-HindIII* fragment into the *BamHI-HindIII* sites of pBluescript II SK.

Destruction of internal restriction sites which are required for further construction is undertaken using the procedure of "inside-outside PCR" described above (Innes *et al.* PCR Protocols: A guide to methods and applications. Academic Press, New York (1990)). In the case of the *phzB* ORF two *SphI* sites are destroyed (one site located upstream of the ORF is left intact). The first of these is destroyed using the unique restriction sites *EcoRI* (left of the *SphI* site to be destroyed) and *BclI* (right of the *SphI* site). For this manipulation to be successful, the DNA to be *BclI* cleaved for the final assembly of the inside-outside PCR product must be produced in a *dam-minus E. coli* host such as SCS110 (Stratagene). For the second *phzB SphI* sites, the selected unique restriction sites are *PstI* and *SpeI*, the latter being beyond the *phzB* ORF in the pBluescript polylinker. The *phzC* ORF has no internal *SphI* sites, and so this procedure is not required for *phzC*. The *phzD* ORF, however, has a single *SphI* site which can be removed using the unique restriction sites *XmaI* and *HindIII* (the *XmaI/SmaI* site of the pBluescript polylinker is no longer present due to the insertion of the ORF between the *BamHI* and *HindIII* sites).

The removal of *SphI* sites from the phenazine biosynthetic genes as described above facilitates their transfer to the pCGN1761SENX vector by amplification using an aminoterminal oligonucleotide primer which incorporates an *SphI* site at the ATG and a carboxyterminal primer which incorporates a restriction site not found in the gene being amplified. The resultant amplified fragment is cleaved with *SphI* the restriction enzyme cutting the carboxyterminal sequence and cloned into pCGN1761SENX. Suitable restriction enzyme sites for incorporation into the carboxyterminal primer are *EcoRI* and *NotI* (for all three ORFs; *NotI* will need checking when sequence complete), and *XhoI* (for *phzB* and *phzD*). Given the requirement for the nucleotide C at position 6 within the *SphI* recognition site, in some cases the second codon of the ORF may require changing so as to start with the nucleotide C. This construction fuses each ORF at its ATG to the *SphI* sites of the translation-optimized vector pCGN1761SENX in operable linkage to the double 35S promoter. After construction is complete the final gene insertions and fusion points are resequenced to ensure that no undesired base changes have occurred.

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By utilizing an aminoterminal oligonucleotide primer which incorporates an *NcoI* site at its ATG instead of an *SphI* site, the three *phz* ORFs can also be easily cloned into the translation-optimized vector pCGN1761NENX. None of the three phenazine biosynthetic gene ORFs carry an *NcoI* site and consequently there is no requirement in this case to destroy internal restriction sites. Primers for the carboxyterminus of the gene are designed as described above and the cloning is undertaken in a similar fashion. Given the requirement for the nucleotide G at position 6 within the *NcoI* recognition site, in some cases the second codon of the ORF may require changing so as to start with the nucleotide G. This construction fuses each ORF at its ATG to the *NcoI* site of pCGN1761NENX in operable linkage to the double 35S promoter.

The expression cassettes of the appropriate pCGN1761-derivative vectors are transferred to transformation vectors. Where possible multiple expression cassettes are transferred to a single transformation vector so as to reduce the number of plant transformations and crosses between transformants which may be required to produce plants expressing all four ORFs and thus producing phenazine.

Expression behind 35S with Chloroplast Targeting

The three phenazine ORFs amplified using oligonucleotides carrying an *SphI* site at their aminoterminal are cloned into the 35S-chloroplast targeted vector pCGN1761/CT. The fusions are made to the *SphI* site located at the cleavage site of the *rbcS* transit peptide. The expression cassettes thus created are transferred to appropriate transformation vectors (see above) and used to generate transgenic plants. As chorismate, the likely precursor for phenazine biosynthesis, is synthesized in the chloroplast, it may be advantageous to express the biosynthetic genes for phenazine in the chloroplast to ensure a ready supply of substrate. Transgenic plants expressing all three ORFs will target all three gene products to the chloroplast and will thus synthesize phenazine in the chloroplast.

Expression behind *rbcS* with Chloroplast Targeting

The three phenazine ORFs amplified using oligonucleotides carrying an *SphI* site at their aminoterminal are cloned into the *rbcS*-chloroplast targeted vector pCGN1761*rbcS*/CT. The fusions are made to the *SphI* site located at the cleavage site of the *rbcS* transit peptide. The expression cassettes thus created are transferred to appropriate

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transformation vectors (see above) and used to generate transgenic plants. As chorismate, the likely precursor for phenazine biosynthesis, is synthesized in the chloroplast, it may be advantageous to express the biosynthetic genes for phenazine in the chloroplast to ensure a ready supply of substrate. Transgenic plants expressing all three ORFs will target all four gene products to the chloroplast and will thus synthesize phenazine in the chloroplast. The expression of the three ORFs will, however, be light induced.

Example 44: Expression of the Non-Ribosomally Synthesized Peptide Antibiotic Gramicidin in Transgenic Plants

The three *Bacillus brevis* gramicidin biosynthetic genes *grsA*, *grsB* and *grsT* have been previously cloned and sequenced (Turgay *et al.* Mol. Microbiol. **6**: 529-546 (1992); Kraetzschmar *et al.* J. Bacteriol. **171**: 5422-5429 (1989)). They are 3296, 13358, and 770 bp in length, respectively. These sequences are also published as GenBank accession numbers X61658 and M29703. The manipulations described here can be undertaken using the publicly available clones published by Turgay *et al.* (*supra*) and Kraetzschmar *et al.* (*supra*), or alternatively from newly isolated clones from *Bacillus brevis* isolated as described herein.

Each of the three ORFs *grsA*, *grsB*, and *grsT* is PCR amplified using oligonucleotides which span the entire coding sequence. The leftward (upstream) oligonucleotide includes an *SstI* site and the rightward (downstream) oligonucleotide includes an *XhoI* site. These restriction sites are not found within any of the three coding sequences and enable the amplified products to be cleaved with *SstI* and *XhoI* for insertion into the corresponding sites of pBluescript II SK. This generates the clones pBL-GRSa, pBLGRSb and pBLGRSt. The CG content of these genes lies between 35 and 38%. Ideally, the coding sequences encoding the three genes may be remade using the techniques referred to in Section K, however it is possible that the unmodified genes may be expressed at high levels in transgenic plants without encountering problems due to their AT content. In any case it may be advantageous to modify the genes to include codons preferred in the appropriate target plant species.

The ORF *grsA* contains no *SphI* site and no *NcoI* site. This gene can be thus amplified from pBLGSRa using an aminoterminal oligonucleotide which incorporates either an *SphI*

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site or an *NcoI* site at the ATG, and a second carboxyterminal oligonucleotide which incorporates an *XhoI* site, thus enabling the amplification product to be cloned directly into pCGN1761SENX or pCGN1761NENX behind the double 35S promoter.

The ORF *grsB* contains no *NcoI* site and therefore this gene can be amplified using an aminoterminal oligonucleotide containing an *NcoI* site in the same way as described above for the *grsA* ORF; the amplified fragment is cleaved with *NcoI* and *XhoI* and ligated into pCGN1761NENX. However, the *grsB* ORF contains three *SphI* sites and these are destroyed to facilitate the subsequent cloning steps. The sites are destroyed using the "inside-outside" PCR technique described above. Unique cloning sites found within the *grsB* gene but not within pBluescript II SK are *EcoN1*, *PfIM1*, and *RsrII*. Either *EcoN1* or *PfIM1* can be used together with *RsrII* to remove the first two sites and *RsrII* can be used together with the *ApaI* site of the pBluescript polylinker to remove the third site. Once these sites have been destroyed (without causing a change in amino acid), the entirety of the *grsB* ORF can be amplified using an aminoterminal oligonucleotide including an *SphI* site at the ATG and a carboxyterminal oligonucleotide incorporating an *XhoI* site. The resultant fragment is cloned into pCGN1761SENX. In order to successfully PCR-amplify fragments of such size, amplification protocols are modified in view of Barnes (1994, Proc. Natl. Acad. Sci USA 91: 2216-2220 (1994)) who describes the high fidelity amplification of large DNA fragments. An alternative approach to the transfer of the *grsB* ORF to pCGN1761SENX without necessitating the destruction of the three *SphI* restriction sites involves the transfer to the *SphI* and *XhoI* cloning sites of pCGN1761SENX of an aminoterminal fragment of *grsB* by amplification from the ATG of the gene using an aminoterminal oligonucleotide which incorporates a *SphI* site at the ATG, and a second oligonucleotide which is adjacent and 3' to the *PfIM1* site in the ORF and which includes an *XhoI* site. Thus the aminoterminal amplified fragment is cleaved with *SphI* and *XhoI* and cloned into pCGN1761SENX. Subsequently the remaining portion of the *grsB* gene is excised from pBLGRSb using *PfIM1* and *XhoI* (which cuts in the pBluescript polylinker) and cloned into the aminoterminal carrying construction cleaved with *PfIM1* and *XhoI* to reconstitute the gene.

The ORF *grsT* contains no *SphI* site and no *NcoI* site. This gene can be thus amplified from pBLGSRT using an aminoterminal oligonucleotide which incorporates either an *SphI* site or an *NcoI* site at the initiating codon which is changed to ATG (from GTG) for

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expression in plants, and a second carboxyterminal oligonucleotide which incorporates an *XhoI* site, thus enabling the amplification product to be cloned directly into pCGN1761SENX or pCGN1761NENX behind the double 35S promoter.

Given the requirement for the nucleotide C at position 6 within the *SphI* recognition site, and the requirement for the nucleotide G at position 6 within the *NcoI* recognition site, in some cases the second codon of the ORF may require changing so as to start with the appropriate nucleotide.

Transgenic plants are created which express all three gramicidin biosynthetic genes as described elsewhere in the specification. Transgenic plants expressing all three genes synthesize gramicidin.

Example 45: Expression of the Ribosomally Synthesized Peptide Lantibiotic Epidermin in Transgenic Plants

The *epiA* ORF encodes the structural unit for epidermin biosynthesis and is approximately 420 bp in length (GenBank Accession No. X07840; Schnell *et al.* Nature 333: 276-278 (1988)). This gene can be subcloned using PCR techniques from the plasmid pTü32 into pBluescript SK II using oligonucleotides carrying the terminal restriction sites *BamHI* (5') and *PstI* (3'). The *epiA* gene sequence has a GC content of 27% and this can be increased using techniques of gene synthesis referred to elsewhere in this specification; this sequence modification may not be essential, however, to ensure high-level expression in plants. Subsequently the *epiA* ORF is transferred to the cloning vector pCGN1761SENX or pCGN1761NENX by PCR amplification of the gene using an aminoterminal oligonucleotide spanning the initiating methionine and carrying an *SphI* site (for cloning into pCGN1761SENX) or an *NcoI* site (for cloning into pCGN1761NENX), together with a carboxyterminal oligonucleotide carrying an *EcoRI*, a *NotI*, or an *XhoI* site for cloning into either pCGN1761SENX or pCGN1761NENX. Given the requirement for the nucleotide C at position 6 within the *SphI* recognition site, and the requirement for the nucleotide G at position 6 within the *NcoI* recognition site, in some cases the second codon of the ORF may require changing so as to start with the appropriate nucleotide.

Using cloning techniques described in this specification or well known in the art, the remaining genes of the *epi* operon (*viz.* *epiB*, *epiC*, *epiD*, *epiQ*, and *epiP*) are subcloned

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from plasmid pTü32 into pBluescript SK II. These genes are responsible for the modification and polymerization of the *epiA*-encoded structural unit and are described in Kupke *et al.* (J. Bacteriol. 174: 5354-5361 (1992)) and Schnell *et al.* (Eur. J. Biochem. 204: 57-68 (1992)). The subcloned ORFs are manipulated for transfer to pCGN1761-derivative vectors as described above. The expression cassettes of the appropriate pCGN1761-derivative vectors are transferred to transformation vectors. Where possible multiple expression cassettes are transferred to a single transformation vector so as to reduce the number of plant transformations and crosses between transformants which may be required to produce plants expressing all required ORFs and thus producing epidermin.

L. Analysis of Transgenic Plants for APS Accumulation

Example 46: Analysis of APS Gene Expression

Expression of APS genes in transgenic plants can be analyzed using standard Northern blot techniques to assess the amount of APS mRNA accumulating in tissues. Alternatively, the quantity of APS gene product can be assessed by Western analysis using antisera raised to APS biosynthetic gene products. Antisera can be raised using conventional techniques and proteins derived from the expression of APS genes in a host such as *E. coli*. To avoid the raising of antisera to multiple gene products from *E. coli* expressing multiple APS genes from multiple ORF operons, the APS biosynthetic genes can be expressed individually in *E. coli*. Alternatively, antisera can be raised to synthetic peptides designed to be homologous or identical to known APS biosynthetic predicted amino acid sequence. These techniques are well known in the art.

Example 47: Analysis of APS Production in Transgenic Plants

For each APS, known protocols are used to detect production of the APS in transgenic plant tissue. These protocols are available in the appropriate APS literature. For pyrrolnitrin, the procedure described in example 11 is used, and for soraphen the procedure described in example 17. For phenazine determination, the procedure described in example 18 can be used. For non-ribosomal peptide antibiotics such as gramicidin S, an appropriate general technique is the assaying of ATP-PP_i exchange. In the case of gramicidin, the *grsA* gene can be assayed by phenylalanine-dependent ATP-PP_i exchange

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and the *grsB* gene can be assayed by proline, valine, ornithine, or leucine-dependent ATP-PP_i exchange. Alternative techniques are described by Gause & Brazhnikova (Lancet 247: 715 (1944)). For ribosomally synthesized peptide antibiotics isolation can be achieved by butanol extraction, dissolving in methanol and diethyl ether, followed by chromatography as described by Allgaier *et al.* for epidermin (Eur. J. Biochem. 160: 9-22 (1986)). For many APSs (*e.g.* pyrrolnitrin, gramicidin, phenazine) appropriate techniques are provided in the Merck Index (Merck & Co., Rahway, NJ (1989)).

M. Assay of Disease Resistance in Transgenic Plants

Transgenic plants expressing APS biosynthetic genes are assayed for resistance to phytopathogens using techniques well known in phytopathology. For foliar pathogens, plants are grown in the greenhouse and at an appropriate stage of development inoculum of a phytopathogen of interest is introduced in an appropriate manner. For soil-borne phytopathogens, the pathogen is normally introduced into the soil before or at the time the seeds are planted. The choice of plant cultivar selected for introduction of the genes will have taken into account relative phytopathogen sensitivity. Thus, it is preferred that the cultivar chosen will be susceptible to most phytopathogens of interest to allow a determination of enhanced resistance.

Assay of Resistance to Foliar Phytopathogens

Example 48: Disease Resistance to Tobacco Foliar Phytopathogens

Transgenic tobacco plants expressing APS genes and shown to produce APS compound are subjected to the following disease tests.

***Phytophthora parasitica*/Black shank** Assays for resistance to *Phytophthora parasitica*, the causative organism of black shank are performed on six-week-old plants grown as described in Alexander *et al.*, Pro. Natl. Acad. Sci. USA 90: 7327-7331. Plants are watered, allowed to drain well, and then inoculated by applying 10 mL of a sporangium suspension (300 sporangia/mL) to the soil. Inoculated plants are kept in a greenhouse maintained at 23-25 C day temperature, and 20-22 C night temperature. The wilt index used for the assay is as follows: 0 = no symptoms; 1 = some sign of wilting, with reduced turgidity; 2 = clear wilting symptoms, but no rotting or stunting; 3 = clear wilting symptoms with stunting, but no apparent stem rot; 4 = severe wilting, with visible stem rot and some damage to root

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system; 5 = as for 4, but plants near death or dead, and with severe reduction of root system. All assays are scored blind on plants arrayed in a random design.

Pseudomonas syringae *Pseudomonas syringae* pv. *tabaci* (strain #551) is injected into the two lower leaves of several 6-7 week old plants at a concentration of 10^6 or 3×10^6 per ml in H₂O. Six individual plants are evaluated at each time point. *Pseudomonas tabaci* infected plants are rated on a 5 point disease severity scale, 5 = 100% dead tissue, 0 = no symptoms. A T-test (LSD) is conducted on the evaluations for each day and the groupings are indicated after the Mean disease rating value. Values followed by the same letter on that day of evaluation are not statistically significantly different.

Cercospora nicotianae A spore suspension of *Cercospora nicotianae* (ATCC #18366) (100,000-150,000 spores per ml) is sprayed to imminent run-off on to the surface of the leaves. The plants are maintained in 100% humidity for five days. Thereafter the plants are misted with H₂O 5-10 times per day. Six individual plants are evaluated at each time point. *Cercospora nicotianae* is rated on a % leaf area showing disease symptoms basis. A T-test (LSD) is conducted on the evaluations for each day and the groupings are indicated after the Mean disease rating value. Values followed by the same letter on that day of evaluation are not statistically significantly different.

Statistical Analyses All tests include non-transgenic plants (six plants per assay, or the same cultivar as the transgenic lines) (Alexander *et al.*, Pro. Natl. Acad. Sci. USA 90: 7327-7331). Pairwise T-tests are performed to compare different genotype and treatment groups for each rating date.

Assay of Resistance to Soil-Borne Phytopathogens

Example 49: Resistance to *Rhizoctonia solani*

Plant assays to determine resistance to *Rhizoctonia solani* are conducted by planting or transplanting seeds or seedlings into naturally or artificially infested soil. To create artificially infested soil, millet, rice, oat, or other similar seeds are first moistened with water, then autoclaved and inoculated with plugs of the fungal phytopathogen taken from an agar plate. When the seeds are fully overgrown with the phytopathogen, they are air-dried and

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ground into a powder. The powder is mixed into soil at a rate experimentally determined to cause disease. Disease may be assessed by comparing stand counts, root lesions ratings, and shoot and root weights of transgenic and non-transgenic plants grown in the infested soil. The disease ratings may also be compared to the ratings of plants grown under the same conditions but without phytopathogen added to the soil.

Example 50: Resistance to *Pseudomonas solanacearum*

Plant assays to determine resistance to *Pseudomonas solanacearum* are conducted by planting or transplanting seeds or seedlings into naturally or artificially infested soil. To create artificially infested soil, bacteria are grown in shake flask cultures, then mixed into the soil at a rate experimentally determined to cause disease. The roots of the plants may need to be slightly wounded to ensure disease development. Disease may be assessed by comparing stand counts, degree of wilting and shoot and root weights of transgenic and non-transgenic plants grown in the infested soil. The disease ratings may also be compared to the ratings of plants grown under the same conditions but without phytopathogen added to the soil.

Example 51: Resistance to Soil-Borne Fungi which are Vectors for Virus Transmission

Many soil-borne *Polymyxa*, *Ospidium* and *Spongospora* species are vectors for the transmission of viruses. These include (1) *Polymyxa betae* which transmits Beet Necrotic Yellow Vein Virus (the causative agent of rhizomania disease) to sugar beet, (2) *Polymyxa graminis* which transmits Wheat Soil-Borne Mosaic Virus to wheat, and Barley Yellow Mosaic Virus and Barley Mild Mosaic Virus to barley, (3) *Ospidium brassicae* which transmits Tobacco Necrosis Virus to tobacco, and (4) *Spongospora subterranea* which transmits Potato Mop Top Virus to potato. Seeds or plants expressing APSs in their roots (e.g. constitutively or under root specific expression) are sown or transplanted in sterile soil and fungal inocula carrying the virus of interest are introduced to the soil. After a suitable time period the transgenic plants are assayed for viral symptoms and accumulation of virus by ELISA and Northern blot. Control experiments involve no inoculation, and inoculation with fungus which does not carry the virus under investigation. The transgenic plant lines under analysis should ideally be susceptible to the virus in order to test the efficacy of the APS-based protection. In the case of viruses such as Barley Mild Mosaic Virus which are both

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Polymyxa-transmitted and mechanically transmissible, a further control is provided by the successful mechanical introduction of the virus into plants which are protected against soil-infection by APS expression in roots.

Resistance to virus-transmitting fungi offered by expression of APSs will thus prevent virus infections of target crops thus improving plant health and yield.

Example 52: Resistance to Nematodes

Transgenic plants expressing APSs are analyzed for resistance to nematodes. Seeds or plants expressing APSs in their roots (e.g. constitutively or under root specific expression) are sown or transplanted in sterile soil and nematode inocula carrying are introduced to the soil. Nematode damage is assessed at an appropriate time point. Root knot nematodes such as *Meloidogyne* spp. are introduced to transgenic tobacco or tomato expressing APSs. Cyst nematodes such as *Heterodera* spp. are introduced to transgenic cereals, potato and sugar beet. Lesion nematodes such as *Pratylenchus* spp. are introduced to transgenic soybean, alfalfa or corn. Reniform nematodes such as *Rotylenchulus* spp. are introduced to transgenic soybean, cotton, or tomato. *Ditylenchus* spp. are introduced to transgenic alfalfa. Detailed techniques for screening for resistance to nematodes are provided in Starr (Ed.; Methods for Evaluating Plant Species for resistance to Plant Parasitic Nematodes, Society of Nematologists, Hyattsville, Maryland (1990))

Examples of Important Phytopathogens in Agricultural Crop Species

Example 53: Disease Resistance in Maize

Transgenic maize plants expressing APS genes and shown to produce APS compound are subjected to the following disease tests. Tests for each phytopathogen are conducted according to standard phytopathological procedures.

Leaf Diseases and Stalk Rots

- (1) Northern Corn Leaf Blight (*Helminthosporium turcicum*† syn. *Exserohilum turcicum*).
- (2) Anthracnose (*Colletotrichum graminicola*†-same as for Stalk Rot)
- (3) Southern Corn Leaf Blight (*Helminthosporium maydis*† syn. *Bipolaris maydis*).
- (4) Eye Spot (*Kabatiella zeae*)
- (5) Common Rust (*Puccinia sorghi*).

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- (6) Southern Rust (*Puccinia polysora*).
- (7) Gray Leaf Spot (*Cercospora zeae-maydis*† and *C. sorghi*)
- (8) Stalk Rots (a complex of two or more of the following pathogens-*Pythium aphanidermatum*†-early, *Erwinia chrysanthemi-zeae*-early, *Colletotrichum graminicola*†, *Diplodia maydis*†, *D. macrospora*, *Gibberella zeae*†, *Fusarium moniliforme*†, *Macrophomina phaseolina*, *Cephalosporium acremonium*)
- (9) Goss' Disease (*Clavibacter nebraskanense*)

Important-Ear Molds

- (1) Gibberella Ear Rot (*Gibberella zeae*†-same as for Stalk Rot)
Aspergillus flavus, *A. parasiticus*. Aflatoxin
- (2) Diplodia Ear Rot (*Diplodia maydis*† and *D. macrospora*-same organisms as for Stalk Rot)
- (3) Head Smut (*Sphacelotheca reiliana*--syn. *Ustilago reiliana*)

Example 54: Disease Resistance in Wheat

Transgenic wheat plants expressing APS genes and shown to produce APS compound are subjected to the following disease tests. Tests for each pathogen are conducted according to standard phytopathological procedures.

- (1) Septoria Diseases (*Septoria tritici*, *S. nodorum*)
- (2) Powdery Mildew (*Erysiphe graminis*)
- (3) Yellow Rust (*Puccinia striiformis*)
- (4) Brown Rust (*Puccinia recondita*, *P. hordei*)
- (5) Others-Brown Foot Rot/Seedling Blight (*Fusarium culmorum* and *Fusarium roseum*), Eyespot (*Pseudocercospora herpotrichoides*), Take-All (*Gaeumannomyces graminis*)
- (6) Viruses (barley yellow mosaic virus, barley yellow dwarf virus, wheat yellow mosaic virus).

N. Assay of Biocontrol Efficacy in Microbial Strains Expressing APS Genes

Example 55: Protection of Cotton against *Rhizoctonia solani*

Assays to determine protection of cotton from infection caused by *Rhizoctonia solani* are conducted by planting seeds treated with the biocontrol strain in naturally or artificially

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infested soil. To create artificially infested soil, millet, rice, oat, or other similar seeds are first moistened with water, then autoclaved and inoculated with plugs of the fungal pathogen taken from an agar plate. When the seeds are fully overgrown with the pathogen, they are air-dried and ground into a powder. The powder is mixed into soil at a rate experimentally determined to cause disease. This infested soil is put into pots, and seeds are placed in furrows 1.5cm deep. The biocontrol strains are grown in shake flasks in the laboratory. The cells are harvested by centrifugation, resuspended in water, and then drenched over the seeds. Control plants are drenched with water only. Disease may be assessed 14 days later by comparing stand counts and root lesions ratings of treated and nontreated seedlings. The disease ratings may also be compared to the ratings of seedlings grown under the same conditions but without pathogen added to the soil.

Example 56: Protection of Potato against *Claviceps michiganese* subsp. *speedonicum*

Claviceps michiganese subsp. *speedonicum* is the causal agent of potato ring rot disease and is typically spread before planting when "seed" potato tubers are knife cut to generate more planting material. Transmission of the pathogen on the surface of the knife results in the inoculation of entire "seed" batches. Assays to determine protection of potato from the causal agent of ring rot disease are conducted by inoculating potato seed pieces with both the pathogen and the biocontrol strain. The pathogen is introduced by first cutting a naturally infected tuber, then using the knife to cut other tubers into seed pieces. Next, the seed pieces are treated with a suspension of biocontrol bacteria or water as a control. Disease is assessed at the end of the growing season by evaluating plant vigor, yield, and number of tubers infected with *Clavibacter*.

O. Isolation of APSs from Organisms Expressing the Cloned Genes

Example 57: Extraction Procedures for APS Isolation

Active APSs can be isolated from the cells or growth medium of wild-type or transformed strains that produces the APS. This can be undertaken using known protocols for the isolation of molecules of known characteristics.

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For example, for APSs which contain multiple benzene rings (pyrrolnitrin and soraphen) cultures are grown for 24 h in 10 ml L broth at an appropriate temperature and then extracted with an equal volume of ethyl acetate. The organic phase is recovered, allowed to evaporated under vacuum and the residue dissolved in 20 l of methanol.

In the case of pyrrolnitrin a further procedure has been used successfully for the extraction of the active antipathogenic compound from the growth medium of the transformed strain producing this antibiotic. This is accomplished by extraction of the medium with 80% acetone followed by removal of the acetone by evaporation and a second extraction with diethyl ether. The diethyl ether is removed by evaporation and the dried extract is resuspended in a small volume of water. Small aliquots of the antibiotic extract applied to small sterile filter paper discs placed on an agar plate will inhibit the growth of *Rhizoctonia solani*, indicating the presence of the active antibiotic compound.

A preferred method for phenazine isolation is described by Thomashow *et al.* (Appl Environ Microbiol 56: 908-912 (1990)). This involves acidifying cultures to pH 2.0 with HCl and extraction with benzene. Benzene fractions are dehydrated with Na₂SO₄ and evaporated to dryness. The residue is redissolved in aqueous 5% NaHCO₃, reextracted with an equal volume of benzene, acidified, partitioned into benzene and redried.

For peptide antibiotics (which are typically hydrophobic) extraction techniques using butanol, methanol, chloroform or hexane are suitable. In the case of gramicidin, isolation can be carried out according to the procedure described by Gause & Brazhnikova (Lancet 247: 715 (1944)). For epidermin, the procedure described by Allgaier *et al.* for epidermin (Eur. J. Biochem. 160: 9-22 (1986)) is suitable and involves butanol extraction, and dissolving in methanol and diethyl ether. For many APSs (e.g. pyrrolnitrin, gramicidin, phenazine) appropriate techniques are provided in the Merck Index (Merck & Co., Rahway, NJ (1989)).

P. Formulation and Use of Isolated Antibiotics

Antifungal formulations can be made using active ingredients which comprise either the isolated APSs or alternatively suspensions or concentrates of cells which produce them. Formulations can be made in liquid or solid form.

Example 58: Liquid Formulation of Antifungal Compositions

In the following examples, percentages of composition are given by weight:

1. Emulsifiable concentrates:	a	b	c
Active ingredient	20%	40%	50%
Calcium dodecylbenzenesulfonate	5%	8%	6%
Castor oil polyethylene glycol ether (36 moles of ethylene oxide)	5%	-	-
Tributylphenol polyethylene glycol ether (30 moles of ethylene oxide)	-	12%	4%
Cyclohexanone	-	15%	20%
Xylene mixture	70%	25 %	20%

Emulsions of any required concentration can be produced from such concentrates by dilution with water.

2. Solutions:	a	b	c	d
Active ingredient	80%	10%	5%	95%
Ethylene glycol monomethyl ether	20%	-	-	-
Polyethylene glycol 400	-	70%	-	-
N-methyl-2-pyrrolidone	-	20 %	-	-
Epoxidised coconut oil	-	-	1%	5%
Petroleum distillate	-	-	94%	-

(boiling range 160-190°)

These solutions are suitable for application in the form of microdrops.

3. Granulates:	a	b
Active ingredient	5%	10%
Kaolin	94%	-
Highly dispersed silicic acid	1%	-
Attapulgit	-	90%

The active ingredient is dissolved in methylene chloride, the solution is sprayed onto the carrier, and the solvent is subsequently evaporated off in vacuo.

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4. Dusts:	a	b
Active ingredient	2%	5%
Highly dispersed silicic acid	1%	5%
Talcum	97%	-
Kaolin	-	90%

Ready-to-use dusts are obtained by intimately mixing the carriers with the active ingredient.

Example 59: Solid Formulation of Antifungal Compositions

In the following examples, percentages of compositions are by weight.

1. Wettable powders:	a	b	c
Active ingredient	20%	60%	75%
Sodium lignosulfonate	5%	5%	-
Sodium lauryl sulfate	3%	-	5%
Sodium diisobutylphenylthalethene sulfonate	-	6%	10 %
Octylphenol polyethylene glycol ether (7-8 moles of ethylene oxide)	-	2%	-
Highly dispersed silicic acid	5%	27%	10%
Kaolin	67%	-	-

The active ingredient is thoroughly mixed with the adjuvants and the mixture is thoroughly ground in a suitable mill, affording wettable powders which can be diluted with water to give suspensions of the desired concentrations.

2. Emulsifiable concentrate:

Active ingredient	10%
Octylphenol polyethylene glycol ether (4-5 moles of ethylene oxide)	3%
Calcium dodecylbenzenesulfonate	3%
Castor oil polyglycol ether (36 moles of ethylene oxide)	4%
Cyclohexanone	30%
Xylene mixture	50%

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Emulsions of any required concentration can be obtained from this concentrate by dilution with water.

3. Dusts:

	a	b
Active ingredient	5%	8%
Talcum	95%	-
Kaolin	-	92%

Ready-to-use dusts are obtained by mixing the active ingredient with the carriers, and grinding the mixture in a suitable mill.

4. Extruder granulate:

Active ingredient	10%
Sodium lignosulfonate	2%
Carboxymethylcellulose	1%
Kaolin	87%

The active ingredient is mixed and ground with the adjuvants, and the mixture is subsequently moistened with water. The mixture is extruded and then dried in a stream of air.

5. Coated granulate:

Active ingredient	3%
Polyethylene glycol 200	3%
Kaolin	94%

The finely ground active ingredient is uniformly applied, in a mixer, to the kaolin moistened with polyethylene glycol. Non-dusty coated granulates are obtained in this manner.

6. Suspension concentrate:

Active ingredient	40%
Ethylene glycol	10%
Nonylphenol polyethylene glycol (15 moles of ethylene oxide)	6%

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Sodium lignosulfonate	10%
Carboxymethylcellulose	1%
37 % aqueous formaldehyde solution	0.2%
Silicone oil in 75 % aqueous emulsion	0.8%
Water	32%

The finely ground active ingredient is intimately mixed with the adjuvants, giving a suspension concentrate from which suspensions of any desired concentration can be obtained by dilution with water.

While the present invention has been described with reference to specific embodiments thereof, it will be appreciated that numerous variations, modifications, and embodiments are possible, and accordingly, all such variations, modifications and embodiments are to be regarded as being within the spirit and scope of the present invention.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

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- (I) TELEX: 962 991

(ii) TITLE OF INVENTION: Genes for the synthesis of antipathogenic substances

(iii) NUMBER OF SEQUENCES: 22

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7000 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (B) STRAIN: single

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 357..2039
- (D) OTHER INFORMATION: /label= ORF1

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 2249..3076
- (D) OTHER INFORMATION: /label= ORF2

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(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 3166..4869
 (D) OTHER INFORMATION: /label= ORF3

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 4894..5985
 (D) OTHER INFORMATION: /label= ORF4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GAATTCCGAC AACGCCGAAG AAGCGCGGAA CCGCTGAAAG AGGAGCAGGA ACTGGAGCAA	60
ACGCTGTCCC AGGTGATCGA CAGCCTGCCA CTGCGCATCG AGGGCOGATG AACAGCATTG	120
GCAAAAGCTG GCGGTGCGCA GTGCGCGAGT GATCCGATCA TTTTGTGATCG GCTCGCCTCT	180
TCAAAATCGG CCGTGGATGA AGTOGACGGC GGACTGATCA GGCGCAAAG AACATGCGCC	240
AAAACCTTCT TTTATAGCGA ATACCTTTGC ACTTCAGAAT GTTAATTOGG AAACGGAATT	300
TGCATCGCTT TTCGGCGAGT CTAGAGTCTC TAACAGCACA TTGATGTGCC TCTTGC	356
ATG GAT GCA CGA AGA CTG GCG GCC TCC OCT CGT CAC AGG CGG CCC GCC	404
Met Asp Ala Arg Arg Leu Ala Ala Ser Pro Arg His Arg Arg Pro Ala	
1 5 10 15	
TTT GAC ACA AGG AGT GTT ATG AAC AAG CCG ATC AAG AAT ATC GTC ATC	452
Phe Asp Thr Arg Ser Val Met Asn Lys Pro Ile Lys Asn Ile Val Ile	
20 25 30	
GTG GGC GGC GGT ACT GCG GGC TGG ATG GCC GCC TCG TAC CTC GTC OGG	500
Val Gly Gly Gly Thr Ala Gly Trp Met Ala Ala Ser Tyr Leu Val Arg	
35 40 45	
GCC CTC CAA CAG CAG GCG AAC ATT ACG CTC ATC GAA TCT GCG GCG ATC	548
Ala Leu Gln Gln Gln Ala Asn Ile Thr Leu Ile Glu Ser Ala Ala Ile	
50 55 60	
CCT OGG ATC GGC GTG GGC GAA GCG ACC ATC CCA AGT TTG CAG AAG GTG	596
Pro Arg Ile Gly Val Gly Glu Ala Thr Ile Pro Ser Leu Gln Lys Val	
65 70 75 80	
TTC TTC GAT TTC CTC GGG ATA CCG GAG OGG GAA TGG ATG CCC CAA GTG	644
Phe Phe Asp Phe Leu Gly Ile Pro Glu Arg Glu Trp Met Pro Gln Val	
85 90 95	
AAC GGC GCG TTC AAG GCC GCG ATC AAG TTC GTG AAT TGG AGA AAG TCT	692
Asn Gly Ala Phe Lys Ala Ala Ile Lys Phe Val Asn Trp Arg Lys Ser	
100 105 110	
CCC GAC CCC TCG CGC GAC GAT CAC TTC TAC CAT TTG TTC GGC AAC GTG	740
Pro Asp Pro Ser Arg Asp Asp His Phe Tyr His Leu Phe Gly Asn Val	

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115	120	125	
CCG AAC TGC GAC GGC GTG Pro Asn Cys Asp Gly Val 130	CCG CTT ACC CAC TAC Pro Leu Thr His Tyr 135	TGG CTG CGC AAG CGC Trp Leu Arg Lys Arg 140	788
GAA CAG GGC TTC CAG CAG Glu Gln Gly Phe Gln Gln 145	CCG ATG GAG TAC GCG Pro Met Glu Tyr Ala 150	TGC TAC CCG CAG CCC Cys Tyr Pro Gln Pro 155	836
GGG GCA CTC GAC GGC AAG Gly Ala Leu Asp Gly Lys 165	CTG GCA CCG TGC CTG Leu Ala Pro Cys Leu 170	TCC GAC GGC ACC CGC Ser Asp Gly Thr Arg 175	884
CAG ATG TCC CAC GCG TGG Gln Met Ser His Ala Trp 180	CAC TTC GAC GCG CAC His Phe Asp Ala His 185	CTG GTG GCC GAC TTC Leu Val Ala Asp Phe 190	932
TTG AAG CGC TGG GCC GTC Leu Lys Arg Trp Ala Val 195	GAG CGC GGG GTG AAC Glu Arg Gly Val Asn 200	CGC GTG GTC GAT GAG Arg Val Val Asp Glu 205	980
GTG GTG GAC GTT CGC CTG Val Val Asp Val Arg Leu 210	AAC AAC CGC GGC TAC Asn Asn Arg Gly Tyr 215	ATC TCC AAC CTG CTC Ile Ser Asn Leu Leu 220	1028
ACC AAG GAG GGG CGG ACG Thr Lys Glu Gly Arg Thr 225	CTG GAG GCG GAC CTG Leu Glu Ala Asp Leu 230	TTC ATC GAC TGC TCC Phe Ile Asp Cys Ser 235	1076
GGC ATG CGG GGG CTC CTG Gly Met Arg Gly Leu Leu 245	ATC AAT CAG GCG CTG Ile Asn Gln Ala Leu 250	AAG GAA CCC TTC ATC Lys Glu Pro Phe Ile 255	1124
GAC ATG TCC GAC TAC CTG Asp Met Ser Asp Tyr Leu 260	CTG CTG TGC GAC AGC Leu Leu Cys Asp Ser 265	GCG GTC GCC AGC GCC Ala Val Ala Ser Ala 270	1172
CCC AAC GAC GAC GCG CGC Pro Asn Asp Asp Ala Arg 275	GAT GGG GTC GAG CCG Asp Gly Val Glu Pro 280	TAC ACC TCC TCG ATC Tyr Thr Ser Ser Ile 285	1220
GCC ATG AAC TCG GGA TGG Ala Met Asn Ser Gly Trp 290	ACC TGG AAG ATT CCG Thr Trp Lys Ile Pro 295	ATG CTG GGC CGG TTC Met Leu Gly Arg Phe 300	1268
GGC AGC GGC TAC GTC TTC Gly Ser Gly Tyr Val Phe 305	TCG AGC CAT TTC ACC Ser Ser His Phe Thr 310	TCG CGC GAC CAG GCC Ser Arg Asp Gln Ala 315	1316
ACC GCC GAC TTC CTC AAA Thr Ala Asp Phe Leu Lys 325	CTC TGG GGC CTC TCG Leu Trp Gly Leu Ser 330	GAC AAT CAG CCG CTC Asp Asn Gln Pro Leu 335	1364
AAC CAG ATC AAG TTC CGG Leu Arg Thr Lys Thr 340	GTC GGG CGC AAC AAG Glu Gly Thr Ser Thr 345	CGG GCG TGG GTC AAC Arg Thr Thr Thr Thr 350	1412

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Asn	Gln	Ile	Lys	Phe	Arg	Val	Gly	Arg	Asn	Lys	Arg	Ala	Trp	Val	Asn	
			340					345					350			
AAC	TGC	GTC	TCG	ATC	GGG	CTG	TCG	TCG	TGC	TTT	CTG	GAG	CCC	CTG	GAA	1460
Asn	Cys	Val	Ser	Ile	Gly	Leu	Ser	Ser	Cys	Phe	Leu	Glu	Pro	Leu	Glu	
		355					360					365				
TCG	ACG	GGG	ATC	TAC	TTC	ATC	TAC	GCG	GCG	CTT	TAC	CAG	CTC	GTG	AAG	1508
Ser	Thr	Gly	Ile	Tyr	Phe	Ile	Tyr	Ala	Ala	Leu	Tyr	Gln	Leu	Val	Lys	
	370					375					380					
CAC	TTC	CCC	GAC	ACC	TCG	TTC	GAC	CCG	CGG	CTG	AGC	GAC	GCT	TTC	AAC	1556
His	Phe	Pro	Asp	Thr	Ser	Phe	Asp	Pro	Arg	Leu	Ser	Asp	Ala	Phe	Asn	
385					390					395					400	
GCC	GAG	ATC	GTC	CAC	ATG	TTC	GAC	GAC	TGC	CGG	GAT	TTC	GTC	CAA	GCG	1604
Ala	Glu	Ile	Val	His	Met	Phe	Asp	Asp	Cys	Arg	Asp	Phe	Val	Gln	Ala	
				405					410					415		
CAC	TAT	TTC	ACC	ACG	TCG	CGC	GAT	GAC	ACG	CCG	TTC	TGG	CTC	GCG	AAC	1652
His	Tyr	Phe	Thr	Thr	Ser	Arg	Asp	Asp	Thr	Pro	Phe	Trp	Leu	Ala	Asn	
			420					425					430			
CGG	CAC	GAC	CTG	CGG	CTC	TCG	GAC	GCC	ATC	AAA	GAG	AAG	GTT	CAG	CGC	1700
Arg	His	Asp	Leu	Arg	Leu	Ser	Asp	Ala	Ile	Lys	Glu	Lys	Val	Gln	Arg	
		435					440					445				
TAC	AAG	GCG	GGG	CTG	CCG	CTG	ACC	ACC	ACG	TCG	TTC	GAC	GAT	TCC	ACG	1748
Tyr	Lys	Ala	Gly	Leu	Pro	Leu	Thr	Thr	Thr	Ser	Phe	Asp	Asp	Ser	Thr	
	450					455					460					
TAC	TAC	GAG	ACC	TTC	GAC	TAC	GAA	TTC	AAG	AAT	TTC	TGG	TTG	AAC	GGC	1796
Tyr	Tyr	Glu	Thr	Phe	Asp	Tyr	Glu	Phe	Lys	Asn	Phe	Trp	Leu	Asn	Gly	
465					470					475					480	
AAC	TAC	TAC	TGC	ATC	TTT	GCC	GGC	TTG	GGC	ATG	CTG	CCC	GAC	CGG	TCG	1844
Asn	Tyr	Tyr	Cys	Ile	Phe	Ala	Gly	Leu	Gly	Met	Leu	Pro	Asp	Arg	Ser	
				485					490					495		
CTG	CCG	CTG	TTG	CAG	CAC	CGA	CCG	GAG	TCG	ATC	GAG	AAA	GCC	GAG	GCG	1892
Leu	Pro	Leu	Leu	Gln	His	Arg	Pro	Glu	Ser	Ile	Glu	Lys	Ala	Glu	Ala	
			500					505					510			
ATG	TTC	GCC	AGC	ATC	CGG	CGC	GAG	GCC	GAG	CGT	CTG	CGC	ACC	AGC	CTG	1940
Met	Phe	Ala	Ser	Ile	Arg	Arg	Glu	Ala	Glu	Arg	Leu	Arg	Thr	Ser	Leu	
		515					520					525				
CCG	ACA	AAC	TAC	GAC	TAC	CTG	CGG	TCG	CTG	CGT	GAC	GGC	GAC	GCG	GGG	1988
Pro	Thr	Asn	Tyr	Asp	Tyr	Leu	Arg	Ser	Leu	Arg	Asp	Gly	Asp	Ala	Gly	
		530				535					540					
CTG	TCG	CGC	GGC	CAG	CGT	GGG	CCG	AAG	CTC	GCA	GCG	CAG	GAA	AGC	CTG	2036
Leu	Ser	Arg	Gly	Gln	Arg	Gly	Pro	Lys	Leu	Ala	Ala	Gln	Glu	Ser	Leu	
545					550					555					560	

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TAGTGGAAACG CACCTTGGAC CGGGTAGGCG TATTCGCGGC CACCCACGCT GCOGTGGGGG	2096
CCTGCGATCC GCTGCAGGCG CGCGCGCTCG TTCTGCAACT GCGGGGCTG AACCGTAACA	2156
AGGAOCTGCC CGGTATCGTC GGCCTGCTGC GCGAGTTCCT TCGGTGCGC GGCTGCOCT	2216
GCGGCTGGGG TTTCGTCGAA GCGCGCGCCG CG ATG CGG GAC ATC GGG TTC TTC Met Arg Asp Ile Gly Phe Phe 1 5	2269
CTG GGG TCG CTC AAG CGC CAC GGA CAT GAG CCC GCG GAG GTG GTG CCC Leu Gly Ser Leu Lys Arg His Gly His Glu Pro Ala Glu Val Val Pro 10 15 20	2317
GGG CTT GAG CCG GTG CTG CTC GAC CTG GCA CGC GCG ACC AAC CTG CCG Gly Leu Glu Pro Val Leu Leu Asp Leu Ala Arg Ala Thr Asn Leu Pro 25 30 35	2365
COG CGC GAG ACG CTC CTG CAT GTG ACG GTC TGG AAC CCC ACG GCG GCC Pro Arg Glu Thr Leu Leu His Val Thr Val Trp Asn Pro Thr Ala Ala 40 45 50 55	2413
GAC GCG CAG CGC AGC TAC ACC GGG CTG CCC GAC GAA GCG CAC CTG CTC Asp Ala Gln Arg Ser Tyr Thr Gly Leu Pro Asp Glu Ala His Leu Leu 60 65 70	2461
GAG AGC GTG CGC ATC TCG ATG GCG GCC CTC GAG GCG GCC ATC GCG TTG Glu Ser Val Arg Ile Ser Met Ala Ala Leu Glu Ala Ala Ile Ala Leu 75 80 85	2509
ACC GTC GAG CTG TTC GAT GTG TCC CTG CGG TCG CCC GAG TTC GCG CAA Thr Val Glu Leu Phe Asp Val Ser Leu Arg Ser Pro Glu Phe Ala Gln 90 95 100	2557
AGG TGC GAC GAG CTG GAA GCC TAT CTG CAG AAA ATG GTC GAA TCG ATC Arg Cys Asp Glu Leu Glu Ala Tyr Leu Gln Lys Met Val Glu Ser Ile 105 110 115	2605
GTC TAC GCG TAC CGC TTC ATC TCG CCG CAG GTC TTC TAC GAT GAG CTG Val Tyr Ala Tyr Arg Phe Ile Ser Pro Gln Val Phe Tyr Asp Glu Leu 120 125 130 135	2653
CGC CCC TTC TAC GAA CCG ATT CGA GTC GGG GGC CAG AGC TAC CTC GGC Arg Pro Phe Tyr Glu Pro Ile Arg Val Gly Gly Gln Ser Tyr Leu Gly 140 145 150	2701
CCC GGT GCC GTA GAG ATG CCC CTC TTC GTG CTG GAG CAC GTC CTC TGG Pro Gly Ala Val Glu Met Pro Leu Phe Val Leu Glu His Val Leu Trp 155 160 165	2749
GGC TCG CAA TCG GAC GAC CAA ACT TAT CGA GAA TTC AAA GAG ACG TAC Gly Ser Gln Ser Asp Asp Gln Thr Tyr Arg Glu Phe Lys Glu Thr Tyr 170 175 180	2797
CTG CCC TAT GTG CTT CCC GCG TAC AGG GCG GTC TAC GCT CGG TTC TCC	2845

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Leu	Pro	Tyr	Val	Leu	Pro	Ala	Tyr	Arg	Ala	Val	Tyr	Ala	Arg	Phe	Ser	
185						190					195					
GGG	GAG	COG	GCG	CTC	ATC	GAC	CGC	GCG	CTC	GAC	GAG	GCG	CGA	GCG	GTC	2893
Gly	Glu	Pro	Ala	Leu	Ile	Asp	Arg	Ala	Leu	Asp	Glu	Ala	Arg	Ala	Val	
200				205					210						215	
GGT	ACG	CGG	GAC	GAG	CAC	GTC	CGG	GCT	GGG	CTG	ACA	GCC	CTC	GAG	OGG	2941
Gly	Thr	Arg	Asp	Glu	His	Val	Arg	Ala	Gly	Leu	Thr	Ala	Leu	Glu	Arg	
			220					225						230		
GTC	TTC	AAG	GTC	CTG	CTG	CGC	TTC	CGG	GCG	CCT	CAC	CTC	AAA	TTG	GCG	2989
Val	Phe	Lys	Val	Leu	Leu	Arg	Phe	Arg	Ala	Pro	His	Leu	Lys	Leu	Ala	
			235					240						245		
GAG	CGG	GCG	TAC	GAA	GTC	GGG	CAA	AGC	GGC	COG	AAA	TOG	GCA	GCG	GGG	3037
Glu	Arg	Ala	Tyr	Glu	Val	Gly	Gln	Ser	Gly	Pro	Lys	Ser	Ala	Ala	Gly	
		250					255					260				
GGT	ACG	CGC	CCA	GCA	TGC	TCG	GTG	AGC	TGC	TCA	CGC	TGACGTATGC				3083
Gly	Thr	Arg	Pro	Ala	Cys	Ser	Val	Ser	Cys	Ser	Arg					
	265					270					275					
CGCGCGGTCC	CGCGTCCGCG	CGCGGCTCGA	CGAATCCTGA	TGCGCGCGAC	CCAGTGTTAT											3143
CTCACAAGGA	GAGTTTGCCC	CC	ATG	ACT	CAG	AAG	AGC	CCC	GCG	AAC	GAA	CAC				3195
			Met	Thr	Gln	Lys	Ser	Pro	Ala	Asn	Glu	His				
			1				5					10				
GAT	AGC	AAT	CAC	TTC	GAC	GTA	ATC	ATC	CTC	GGC	TCG	GGC	ATG	TCC	GGC	3243
Asp	Ser	Asn	His	Phe	Asp	Val	Ile	Ile	Leu	Gly	Ser	Gly	Met	Ser	Gly	
			15					20						25		
ACC	CAG	ATG	GGG	GCC	ATC	TTG	GCC	AAA	CAA	CAG	TTT	CGC	GTG	CTG	ATC	3291
Thr	Gln	Met	Gly	Ala	Ile	Leu	Ala	Lys	Gln	Gln	Phe	Arg	Val	Leu	Ile	
			30					35						40		
ATC	GAG	GAG	TCG	TCG	CAC	CCG	CGG	TTC	ACG	ATC	GGC	GAA	TCG	TCG	ATC	3339
Ile	Glu	Glu	Ser	Ser	His	Pro	Arg	Phe	Thr	Ile	Gly	Glu	Ser	Ser	Ile	
		45					50					55				
CCC	GAG	ACG	TCT	CTT	ATG	AAC	CGC	ATC	ATC	GCT	GAT	CGC	TAC	GGC	ATT	3387
Pro	Glu	Thr	Ser	Leu	Met	Asn	Arg	Ile	Ile	Ala	Asp	Arg	Tyr	Gly	Ile	
		60				65					70					
CCG	GAG	CTC	GAC	CAC	ATC	ACG	TCG	TTT	TAT	TCG	ACG	CAA	CGT	TAC	GTC	3435
Pro	Glu	Leu	Asp	His	Ile	Thr	Ser	Phe	Tyr	Ser	Thr	Gln	Arg	Tyr	Val	
		75			80					85					90	
GCG	TCG	AGC	ACG	GGC	ATT	AAG	CGC	AAC	TTC	GGC	TTC	GTG	TTC	CAC	AAG	3483
Ala	Ser	Ser	Thr	Gly	Ile	Lys	Arg	Asn	Phe	Gly	Phe	Val	Phe	His	Lys	
			95						100					105		
CCC	GGC	CAG	GAG	CAC	GAC	CCG	AAG	GAG	TTC	ACC	CAG	TGC	GTC	ATT	CCC	3531
Pro	Gly	Gln	Glu	His	Asp	Pro	Lys	Glu	Phe	Thr	Gln	Cys	Val	Ile	Pro	

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110	115	120	
GAG CTG CCG TGG GGG CCG GAG AGC CAT TAT TAC CGG CAA GAC GTC GAC Glu Leu Pro Trp Gly Pro Glu Ser His Tyr Tyr Arg Gln Asp Val Asp 125 130 135			3579
GCC TAC TTG TTG CAA GCC GCC ATT AAA TAC GGC TGC AAG GTC CAC CAG Ala Tyr Leu Leu Gln Ala Ala Ile Lys Tyr Gly Cys Lys Val His Gln 140 145 150			3627
AAA ACT ACC GTG ACC GAA TAC CAC GCC GAT AAA GAC GGC GTC GCG GTG Lys Thr Thr Val Thr Glu Tyr His Ala Asp Lys Asp Gly Val Ala Val 155 160 165 170			3675
ACC ACC GCC CAG GGC GAA CCG TTC ACC GGC CCG TAC ATG ATC GAC TGC Thr Thr Ala Gln Gly Glu Arg Phe Thr Gly Arg Tyr Met Ile Asp Cys 175 180 185			3723
GGA GGA CCT CGC GCG CCG CTC GCG ACC AAG TTC AAG CTC CGC GAA GAA Gly Gly Pro Arg Ala Pro Leu Ala Thr Lys Phe Lys Leu Arg Glu Glu 190 195 200			3771
CCG TGT CGC TTC AAG ACG CAC TCG CGC AGC CTC TAC ACG CAC ATG CTC Pro Cys Arg Phe Lys Thr His Ser Arg Ser Leu Tyr Thr His Met Leu 205 210 215			3819
GGG GTC AAG CCG TTC GAC GAC ATC TTC AAG GTC AAG GGG CAG CGC TGG Gly Val Lys Pro Phe Asp Asp Ile Phe Lys Val Lys Gly Gln Arg Trp 220 225 230			3867
CGC TGG CAC GAG GGG ACC TTG CAC CAC ATG TTC GAG GGC GGC TGG CTC Arg Trp His Glu Gly Thr Leu His His Met Phe Glu Gly Gly Trp Leu 235 240 245 250			3915
TGG GTG ATT CCG TTC AAC AAC CAC CCG CGG TCG ACC AAC AAC CTG GTG Trp Val Ile Pro Phe Asn Asn His Pro Arg Ser Thr Asn Asn Leu Val 255 260 265			3963
AGC GTC GGC CTG CAG CTC GAC CCG CGT GTC TAC CCG AAA ACC GAC ATC Ser Val Gly Leu Gln Leu Asp Pro Arg Val Tyr Pro Lys Thr Asp Ile 270 275 280			4011
TCC GCA CAG CAG GAA TTC GAT GAG TTC CTC GCG CGG TTC CCG AGC ATC Ser Ala Gln Gln Glu Phe Asp Glu Phe Leu Ala Arg Phe Pro Ser Ile 285 290 295			4059
GGG GCT CAG TTC CCG GAC GCC GTG CCG GTG CGC GAC TGG GTC AAG ACC Gly Ala Gln Phe Arg Asp Ala Val Pro Val Arg Asp Trp Val Lys Thr 300 305 310			4107
GAC CGC CTG CAA TTC TCG TCG AAC GCC TGC GTC GGC GAC CGC TAC TGC Asp Arg Leu Gln Phe Ser Ser Asn Ala Cys Val Gly Asp Arg Tyr Cys 315 320 325 330			4155
CTG ATG CTG CAC GCG AAC GGC TTC ATC GAC CCG CTC TTC TCC CGG GGG			4203

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Leu	Met	Leu	His	Ala	Asn	Gly	Phe	Ile	Asp	Pro	Leu	Phe	Ser	Arg	Gly	
				335					340					345		
CTG	GAA	AAC	ACC	GCG	GTG	ACC	ATC	CAC	GCG	CTC	GCG	GCG	CGC	CTC	ATC	4251
Leu	Glu	Asn	Thr	Ala	Val	Thr	Ile	His	Ala	Leu	Ala	Ala	Arg	Leu	Ile	
			350					355					360			
AAG	GCG	CTG	CGC	GAC	GAC	GAC	TTC	TCC	CCC	GAG	CGC	TTC	GAG	TAC	ATC	4299
Lys	Ala	Leu	Arg	Asp	Asp	Asp	Phe	Ser	Pro	Glu	Arg	Phe	Glu	Tyr	Ile	
		365					370					375				
GAG	CGC	CTG	CAG	CAA	AAG	CTT	TTG	GAC	CAC	AAC	GAC	GAC	TTC	GTC	AGC	4347
Glu	Arg	Leu	Gln	Gln	Lys	Leu	Leu	Asp	His	Asn	Asp	Asp	Phe	Val	Ser	
	380					385					390					
TGC	TGC	TAC	ACG	GCG	TTC	TCG	GAC	TTC	CGC	CTA	TGG	GAC	GCG	TTC	CAC	4395
Cys	Cys	Tyr	Thr	Ala	Phe	Ser	Asp	Phe	Arg	Leu	Trp	Asp	Ala	Phe	His	
395					400					405					410	
AGG	CTG	TGG	GCG	GTC	GGC	ACC	ATC	CTC	GGG	CAG	TTC	CGG	CTC	GTG	CAG	4443
Arg	Leu	Trp	Ala	Val	Gly	Thr	Ile	Leu	Gly	Gln	Phe	Arg	Leu	Val	Gln	
				415					420					425		
GCC	CAC	GCG	AGG	TTC	CGC	GCG	TCG	CGC	AAC	GAG	GGC	GAC	CTC	GAT	CAC	4491
Ala	His	Ala	Arg	Phe	Arg	Ala	Ser	Arg	Asn	Glu	Gly	Asp	Leu	Asp	His	
			430					435					440			
CTC	GAC	AAC	GAC	CCT	CCG	TAT	CTC	GGA	TAC	CTG	TGC	GCG	GAC	ATG	GAG	4539
Leu	Asp	Asn	Asp	Pro	Pro	Tyr	Leu	Gly	Tyr	Leu	Cys	Ala	Asp	Met	Glu	
		445					450					455				
GAG	TAC	TAC	CAG	TTG	TTC	AAC	GAC	GCC	AAA	GCC	GAG	GTC	GAG	GCC	GTG	4587
Glu	Tyr	Tyr	Gln	Leu	Phe	Asn	Asp	Ala	Lys	Ala	Glu	Val	Glu	Ala	Val	
	460					465					470					
AGT	GCC	GGG	CGC	AAG	CCG	GCC	GAT	GAG	GCC	GCG	GCG	CGG	ATT	CAC	GCC	4635
Ser	Ala	Gly	Arg	Lys	Pro	Ala	Asp	Glu	Ala	Ala	Ala	Arg	Ile	His	Ala	
475					480				485						490	
CTC	ATT	GAC	GAA	CGA	GAC	TTC	GCC	AAG	CCG	ATG	TTC	GGC	TTC	GGG	TAC	4683
Leu	Ile	Asp	Glu	Arg	Asp	Phe	Ala	Lys	Pro	Met	Phe	Gly	Phe	Gly	Tyr	
				495				500						505		
TGC	ATC	ACC	GGG	GAC	AAG	CCG	CAG	CTC	AAC	AAC	TCG	AAG	TAC	AGC	CTG	4731
Cys	Ile	Thr	Gly	Asp	Lys	Pro	Gln	Leu	Asn	Asn	Ser	Lys	Tyr	Ser	Leu	
			510					515					520			
CTG	CCG	GCG	ATG	GGG	CTG	ATG	TAC	TGG	ACG	CAA	ACC	CGC	GCG	CCG	GCA	4779
Leu	Pro	Ala	Met	Arg	Leu	Met	Tyr	Trp	Thr	Gln	Thr	Arg	Ala	Pro	Ala	
		525					530					535				
GAG	GTG	AAA	AAG	TAC	TTC	GAC	TAC	AAC	CCG	ATG	TTC	GCG	CTG	CTC	AAG	4827
Glu	Val	Lys	Lys	Tyr	Phe	Asp	Tyr	Asn	Pro	Met	Phe	Ala	Leu	Leu	Lys	
	540					545					550					

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GCG TAC ATC ACG ACC CGC ATC GGC CTG GCG CTG AAG AAG TAGCGCTCG Ala Tyr Ile Thr Thr Arg Ile Gly Leu Ala Leu Lys Lys 555 560 565	4876
ACGACGACAT AAAAACG ATG AAC GAC ATT CAA TTG GAT CAA GCG AGC GTC Met Asn Asp Ile Gln Leu Asp Gln Ala Ser Val 1 5 10	4926
AAG AAG CGT CCC TCG GGC GCG TAC GAC GCA ACC ACG GCG CTG GCC GCG Lys Lys Arg Pro Ser Gly Ala Tyr Asp Ala Thr Thr Arg Leu Ala Ala 15 20 25	4974
AGC TGG TAC GTC GCG ATG CGC TCC AAC GAG CTC AAG GAC AAG CCG ACC Ser Trp Tyr Val Ala Met Arg Ser Asn Glu Leu Lys Asp Lys Pro Thr 30 35 40	5022
GAG TTG ACG CTC TTC GGC CGT CCG TGC GTG GCG TGG CCG GGA GCC ACG Glu Leu Thr Leu Phe Gly Arg Pro Cys Val Ala Trp Arg Gly Ala Thr 45 50 55	5070
GGG CCG GCC GTG GTG ATG GAC CGC CAC TGC TCG CAC CTG GGC GCG AAC Gly Arg Ala Val Val Met Asp Arg His Cys Ser His Leu Gly Ala Asn 60 65 70 75	5118
CTG GCT GAC GGG CCG ATC AAG GAC GGG TGC ATC CAG TGC CCG TTT CAC Leu Ala Asp Gly Arg Ile Lys Asp Gly Cys Ile Gln Cys Pro Phe His 80 85 90	5166
CAC TGG CCG TAC GAC GAA CAG GGC CAG TGC GTT CAC ATC CCC GGC CAT His Trp Arg Tyr Asp Glu Gln Gly Gln Cys Val His Ile Pro Gly His 95 100 105	5214
AAC CAG GCG GTG CCG CAG CTG GAG CCG GTG CCG CCG GGG GCG CGT CAG Asn Gln Ala Val Arg Gln Leu Glu Pro Val Pro Arg Gly Ala Arg Gln 110 115 120	5262
CCG ACG TTG GTC ACC GCC GAG CGA TAC GGC TAC GTG TGG GTC TGG TAC Pro Thr Leu Val Thr Ala Glu Arg Tyr Gly Tyr Val Trp Val Trp Tyr 125 130 135	5310
GGC TCC CCG CTG CCG CTG CAC CCG CTG CCC GAA ATC TCC GCG GCC GAT Gly Ser Pro Leu Pro Leu His Pro Leu Pro Glu Ile Ser Ala Ala Asp 140 145 150 155	5358
GTC GAC AAC GGC GAC TTT ATG CAC CTG CAC TTC GCG TTC GAG ACG ACC Val Asp Asn Gly Asp Phe Met His Leu His Phe Ala Phe Glu Thr Thr 160 165 170	5406
ACG GCG GTC TTG CCG ATC GTC GAG AAC TTC TAC GAC GCG CAG CAC GCA Thr Ala Val Leu Arg Ile Val Glu Asn Phe Tyr Asp Ala Gln His Ala 175 180 185	5454
ACC CCG GTG CAC GCA CTC CCG ATC TCG GCC TTC GAA CTC AAG CTC TTC Thr Pro Val His Ala Leu Pro Ile Ser Ala Phe Glu Leu Lys Leu Phe 190 195 200	5502

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GAC Asp 205	GAT Asp 205	TGG Trp 205	CGC Arg 205	CAG Gln 205	TGG Trp 210	COG Pro 210	GAG Glu 210	GTT Val 210	GAG Glu 215	TCG Ser 215	CTG Leu 215	GCC Ala 215	CTG Leu 215	GCG Ala 215	GGC Gly 215	5550
GCG Ala 220	TGG Trp 220	TTC Phe 220	GGT Gly 225	GCC Ala 225	GGG Gly 225	ATC Ile 225	GAC Asp 230	TTC Phe 230	ACC Thr 230	GTG Val 230	GAC Asp 235	CGG Arg 235	TAC Tyr 235	TTC Phe 235	GGC Gly 235	5598
CCC Pro 240	CTC Leu 240	GCG Gly 240	ATG Met 240	CTG Leu 240	TCA Ser 240	CGC Arg 245	GCG Ala 245	CTC Leu 245	GGC Gly 245	CTG Leu 250	AAC Asn 250	ATG Met 250	TCG Ser 250	CAG Gln 250	ATG Met 250	5646
AAC Asn 255	CTG Leu 255	CAC His 255	TTC Phe 255	GAT Asp 255	GGC Gly 260	TAC Tyr 260	CCC Pro 260	GGC Gly 260	GGG Gly 265	TGC Cys 265	GTC Val 265	ATG Met 265	ACC Thr 265	GTC Val 265	GCC Ala 265	5694
CTG Leu 270	GAC Asp 270	GGA Gly 270	GAC Asp 275	GTC Val 275	AAA Lys 275	TAC Tyr 275	AAG Lys 275	CTG Leu 280	CTC Leu 280	CAG Gln 280	TGT Cys 280	GTG Val 280	ACG Thr 280	COG Pro 280	GTG Val 280	5742
AGC Ser 285	GAA Glu 285	GGC Gly 285	AAG Lys 290	AAC Asn 290	GTC Val 290	ATG Met 290	CAC His 295	ATG Met 295	CTC Leu 295	ATC Ile 295	TCG Ser 295	ATC Ile 295	AAG Lys 295	AAG Lys 295	GTG Val 295	5790
GGC Gly 300	GGC Gly 300	ATC Ile 305	CTG Leu 305	CTC Leu 305	CGC Arg 305	GCG Ala 310	ACC Thr 310	GAC Asp 310	TTC Phe 310	GTG Val 310	CTG Leu 315	TTC Phe 315	GGG Gly 315	CTG Leu 315	CAG Gln 315	5838
ACC Thr 320	AGG Arg 320	CAG Gln 320	GCC Ala 320	GCG Ala 320	GGG Gly 320	TAC Tyr 325	GAC Asp 325	GTC Val 325	AAA Lys 325	ATC Ile 325	TGG Trp 330	AAC Asn 330	GGA Gly 330	ATG Met 330	AAG Lys 330	5886
CCG Pro 335	GAC Asp 335	GGC Gly 335	GGC Gly 335	GCG Ala 340	TAC Tyr 340	AGC Ser 340	AAG Lys 340	TAC Tyr 340	GAC Asp 345	AAG Lys 345	CTC Leu 345	GTG Val 345	CTC Leu 345	AAG Lys 345		5934
TAC Tyr 350	CGG Arg 350	GCG Ala 350	TTC Phe 355	TAT Tyr 355	CGA Arg 355	GGC Gly 355	TGG Trp 355	GTC Val 360	GAC Asp 360	CGC Arg 360	GTC Val 360	GCA Ala 360	AGT Ser 360	GAG Glu 360	CGG Arg 360	5982
TGATGCGTGA	AGCCGAGCOG	CTCTCGACCG	CGTCGCTGCG	CCAGGCGCTC	GCGAACCTGG											6042
CGAGCGGGGT	GACGATCACG	GCTACGGCG	CGCGGGGCC	GCTTGGGCTC	GCGGCCACCA											6102
GCTTCGTGTC	GGAGTCGCTC	TTTGOGAGGT	ATTTCATGACT	ATCTGGCTGT	TGCAACTCGT											6162
GCTGGTGATC	GCGCTCTGCA	ACGTCTGOGG	CGCATTGCC	GAAOGGCTCG	GOCAGTGOGC											6222
GGTCATCGGC	GAGATCGOGG	COGGTTTGCT	GTGGGGGCG	TCGCTGTTTG	GCGTGATCGC											6282
ACCGAGTTTC	TACGACCTGT	TGTTGCGGCC	CCAGGTGCTG	TCAGOGATGG	CGCAAGTCAG											6342
CGAAGTCGGC	CTGGTACTGC	TGATGTTCCA	GGTCGGCCTG	CATATGGAGT	TGGGCGAGAC											6402

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GCTGCGGAC AAGCGCTGGC GCATGCOOCT OGOGATOGCA GCGGGGCGGC TCGTOGCACC 6462
GGCCGGGATC GGCAATGATG TOGCCATOGT TIOGAAAGGC ACGCTCGCCA GCGACGGGCC 6522
GGCGCTGCCC TATGTGCTCT TCTGOGGTGT CGCACTTGCG GTATOGGCGG TGOOGGTGAT 6582
GGCGCGCATC ATCGAOGACC TGGAGCTCAG OGCCATGGTG GCGCGCGGGC ACGCAATGTC 6642
TGCCGGGATG CTGACGGATG CGCTOGGATG GATGCTGCTT GCAACGATTG OCTCGCTATC 6702
GAGCGGGCCC GGCTGGGCAT TTGCGGCGAT GCTCGTCAGC CTGCTOGCGT ATCTGGTGCT 6762
GTGCGGCGTG CTGGTGCGCT TCGTGGTTTG ACGACCCCTT GCGGGGCTCG CGTCGACCGC 6822
GCATGCGACG CGCGACCGCT TGGCOGTGTT GTTCTGCTTC GTAATGTTGT CGGCACTCGC 6882
GAGTGGCTG ATCGGATTCC ATAGCGCTTT TGGGCGACTT GCGGGGCGC TGTTOGTGCG 6942
CCGGTGCCC GCGTGGCGA AGGAGTGGCG CGACAACTG GAAGGTTTCG TCAAGCTT 7000

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 560 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```

Met Asp Ala Arg Arg Leu Ala Ala Ser Pro Arg His Arg Arg Pro Ala
 1             5             10             15
Phe Asp Thr Arg Ser Val Met Asn Lys Pro Ile Lys Asn Ile Val Ile
      20             25             30
Val Gly Gly Gly Thr Ala Gly Trp Met Ala Ala Ser Tyr Leu Val Arg
      35             40             45
Ala Leu Gln Gln Gln Ala Asn Ile Thr Leu Ile Glu Ser Ala Ala Ile
      50             55             60
Pro Arg Ile Gly Val Gly Glu Ala Thr Ile Pro Ser Leu Gln Lys Val
      65             70             75             80
Phe Phe Asp Phe Leu Gly Ile Pro Glu Arg Glu Trp Met Pro Gln Val
      85             90             95
Asn Gly Ala Phe Lys Ala Ala Ile Lys Phe Val Asn Trp Arg Lys Ser
      100            105            110
Pro Asp Pro Ser Arg Asp Asp His Phe Tyr His Leu Phe Gly Asn Val
      115            120            125

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Pro Asn Cys Asp Gly Val Pro Leu Thr His Tyr Trp Leu Arg Lys Arg
 130 135 140
 Glu Gln Gly Phe Gln Gln Pro Met Glu Tyr Ala Cys Tyr Pro Gln Pro
 145 150 155 160
 Gly Ala Leu Asp Gly Lys Leu Ala Pro Cys Leu Ser Asp Gly Thr Arg
 165 170 175
 Gln Met Ser His Ala Trp His Phe Asp Ala His Leu Val Ala Asp Phe
 180 185 190
 Leu Lys Arg Trp Ala Val Glu Arg Gly Val Asn Arg Val Val Asp Glu
 195 200 205
 Val Val Asp Val Arg Leu Asn Asn Arg Gly Tyr Ile Ser Asn Leu Leu
 210 215 220
 Thr Lys Glu Gly Arg Thr Leu Glu Ala Asp Leu Phe Ile Asp Cys Ser
 225 230 235 240
 Gly Met Arg Gly Leu Leu Ile Asn Gln Ala Leu Lys Glu Pro Phe Ile
 245 250 255
 Asp Met Ser Asp Tyr Leu Leu Cys Asp Ser Ala Val Ala Ser Ala Val
 260 265 270
 Pro Asn Asp Asp Ala Arg Asp Gly Val Glu Pro Tyr Thr Ser Ser Ile
 275 280 285
 Ala Met Asn Ser Gly Trp Thr Trp Lys Ile Pro Met Leu Gly Arg Phe
 290 295 300
 Gly Ser Gly Tyr Val Phe Ser Ser His Phe Thr Ser Arg Asp Gln Ala
 305 310 315 320
 Thr Ala Asp Phe Leu Lys Leu Trp Gly Leu Ser Asp Asn Gln Pro Leu
 325 330 335
 Asn Gln Ile Lys Phe Arg Val Gly Arg Asn Lys Arg Ala Trp Val Asn
 340 345 350
 Asn Cys Val Ser Ile Gly Leu Ser Ser Cys Phe Leu Glu Pro Leu Glu
 355 360 365
 Ser Thr Gly Ile Tyr Phe Ile Tyr Ala Ala Leu Tyr Gln Leu Val Lys
 370 375 380
 His Phe Pro Asp Thr Ser Phe Asp Pro Arg Leu Ser Asp Ala Phe Asn
 385 390 395 400
 Ala Glu Ile Val His Met Phe Asp Asp Cys Arg Asp Ph Val Gln Ala
 405 410 415

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His Tyr Phe Thr Thr Ser Arg Asp Asp Thr Pro Phe Trp Leu Ala Asn
 420 425 430
 Arg His Asp Leu Arg Leu Ser Asp Ala Ile Lys Glu Lys Val Gln Arg
 435 440 445
 Tyr Lys Ala Gly Leu Pro Leu Thr Thr Thr Ser Phe Asp Asp Ser Thr
 450 455 460
 Tyr Tyr Glu Thr Phe Asp Tyr Glu Phe Lys Asn Phe Trp Leu Asn Gly
 465 470 475 480
 Asn Tyr Tyr Cys Ile Phe Ala Gly Leu Gly Met Leu Pro Asp Arg Ser
 485 490 495
 Leu Pro Leu Leu Gln His Arg Pro Glu Ser Ile Glu Lys Ala Glu Ala
 500 505 510
 Met Phe Ala Ser Ile Arg Arg Glu Ala Glu Arg Leu Arg Thr Ser Leu
 515 520 525
 Pro Thr Asn Tyr Asp Tyr Leu Arg Ser Leu Arg Asp Gly Asp Ala Gly
 530 535 540
 Leu Ser Arg Gly Gln Arg Gly Pro Lys Leu Ala Ala Gln Glu Ser Leu
 545 550 555 560

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 275 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Met Arg Asp Ile Gly Phe Phe Leu Gly Ser Leu Lys Arg His Gly His
 1 5 10 15
 Glu Pro Ala Glu Val Val Pro Gly Leu Glu Pro Val Leu Leu Asp Leu
 20 25 30
 Ala Arg Ala Thr Asn Leu Pro Pro Arg Glu Thr Leu Leu His Val Thr
 35 40 45
 Val Trp Asn Pro Thr Ala Ala Asp Ala Gln Arg Ser Tyr Thr Gly Leu
 50 55 60
 Pro Asp Glu Ala His Leu Leu Glu Ser Val Arg Ile Ser Met Ala Ala
 65 70 75 80
 Leu Glu Ala Ala Ile Ala Leu Thr Val Glu Leu Phe Asp Val Ser Leu

85

90

95

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) **LENGTH:** 567 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met	Thr	Gln	Lys	Ser	Pro	Ala	Asn	Glu	His	Asp	Ser	Asn	His	Phe	Asp
1				5					10					15	
Val	Ile	Ile	Leu	Gly	Ser	Gly	Met	Ser	Gly	Thr	Gln	Met	Gly	Ala	Ile
			20					25					30		

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Leu Ala Lys Gln Gln Phe Arg Val Leu Ile Ile Glu Glu Ser Ser His
 35 40 45
 Pro Arg Phe Thr Ile Gly Glu Ser Ser Ile Pro Glu Thr Ser Leu Met
 50 55 60
 Asn Arg Ile Ile Ala Asp Arg Tyr Gly Ile Pro Glu Leu Asp His Ile
 65 70 75 80
 Thr Ser Phe Tyr Ser Thr Gln Arg Tyr Val Ala Ser Ser Thr Gly Ile
 85 90 95
 Lys Arg Asn Phe Gly Phe Val Phe His Lys Pro Gly Gln Glu His Asp
 100 105 110
 Pro Lys Glu Phe Thr Gln Cys Val Ile Pro Glu Leu Pro Trp Gly Pro
 115 120 125
 Glu Ser His Tyr Tyr Arg Gln Asp Val Asp Ala Tyr Leu Leu Gln Ala
 130 135 140
 Ala Ile Lys Tyr Gly Cys Lys Val His Gln Lys Thr Thr Val Thr Glu
 145 150 155 160
 Tyr His Ala Asp Lys Asp Gly Val Ala Val Thr Thr Ala Gln Gly Glu
 165 170 175
 Arg Phe Thr Gly Arg Tyr Met Ile Asp Cys Gly Gly Pro Arg Ala Pro
 180 185 190
 Leu Ala Thr Lys Phe Lys Leu Arg Glu Glu Pro Cys Arg Phe Lys Thr
 195 200 205
 His Ser Arg Ser Leu Tyr Thr His Met Leu Gly Val Lys Pro Phe Asp
 210 215 220
 Asp Ile Phe Lys Val Lys Gly Gln Arg Trp Arg Trp His Glu Gly Thr
 225 230 235 240
 Leu His His Met Phe Glu Gly Gly Trp Leu Trp Val Ile Pro Phe Asn
 245 250 255
 Asn His Pro Arg Ser Thr Asn Asn Leu Val Ser Val Gly Leu Gln Leu
 260 265 270
 Asp Pro Arg Val Tyr Pro Lys Thr Asp Ile Ser Ala Gln Gln Glu Phe
 275 280 285
 Asp Glu Phe Leu Ala Arg Phe Pro Ser Ile Gly Ala Gln Phe Arg Asp
 290 295 300
 Ala Val Pro Val Arg Asp Trp Val Lys Thr Asp Arg Leu Gln Phe Ser
 305 310 315 320

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Ser Asn Ala Cys Val Gly Asp Arg Tyr Cys Leu Met Leu His Ala Asn
 325 330 335
 Gly Phe Ile Asp Pro Leu Phe Ser Arg Gly Leu Glu Asn Thr Ala Val
 340 345 350
 Thr Ile His Ala Leu Ala Ala Arg Leu Ile Lys Ala Leu Arg Asp Asp
 355 360 365
 Asp Phe Ser Pro Glu Arg Phe Glu Tyr Ile Glu Arg Leu Gln Gln Lys
 370 375 380
 Leu Leu Asp His Asn Asp Asp Phe Val Ser Cys Cys Tyr Thr Ala Phe
 385 390 395 400
 Ser Asp Phe Arg Leu Trp Asp Ala Phe His Arg Leu Trp Ala Val Gly
 405 410 415
 Thr Ile Leu Gly Gln Phe Arg Leu Val Gln Ala His Ala Arg Phe Arg
 420 425 430
 Ala Ser Arg Asn Glu Gly Asp Leu Asp His Leu Asp Asn Asp Pro Pro
 435 440 445
 Tyr Leu Gly Tyr Leu Cys Ala Asp Met Glu Glu Tyr Tyr Gln Leu Phe
 450 455 460
 Asn Asp Ala Lys Ala Glu Val Glu Ala Val Ser Ala Gly Arg Lys Pro
 465 470 475 480
 Ala Asp Glu Ala Ala Ala Arg Ile His Ala Leu Ile Asp Glu Arg Asp
 485 490 495
 Phe Ala Lys Pro Met Phe Gly Phe Gly Tyr Cys Ile Thr Gly Asp Lys
 500 505 510
 Pro Gln Leu Asn Asn Ser Lys Tyr Ser Leu Leu Pro Ala Met Arg Leu
 515 520 525
 Met Tyr Trp Thr Gln Thr Arg Ala Pro Ala Glu Val Lys Lys Tyr Phe
 530 535 540
 Asp Tyr Asn Pro Met Phe Ala Leu Leu Lys Ala Tyr Ile Thr Thr Arg
 545 550 555 560
 Ile Gly Leu Ala Leu Lys Lys
 565

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 363 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```

Met Asn Asp Ile Gln Leu Asp Gln Ala Ser Val Lys Lys Arg Pro Ser
 1           5           10           15

Gly Ala Tyr Asp Ala Thr Thr Arg Leu Ala Ala Ser Trp Tyr Val Ala
          20           25           30

Met Arg Ser Asn Glu Leu Lys Asp Lys Pro Thr Glu Leu Thr Leu Phe
          35           40           45

Gly Arg Pro Cys Val Ala Trp Arg Gly Ala Thr Gly Arg Ala Val Val
          50           55           60

Met Asp Arg His Cys Ser His Leu Gly Ala Asn Leu Ala Asp Gly Arg
 65           70           75           80

Ile Lys Asp Gly Cys Ile Gln Cys Pro Phe His His Trp Arg Tyr Asp
          85           90           95

Glu Gln Gly Gln Cys Val His Ile Pro Gly His Asn Gln Ala Val Arg
          100          105          110

Gln Leu Glu Pro Val Pro Arg Gly Ala Arg Gln Pro Thr Leu Val Thr
          115          120          125

Ala Glu Arg Tyr Gly Tyr Val Trp Val Trp Tyr Gly Ser Pro Leu Pro
          130          135          140

Leu His Pro Leu Pro Glu Ile Ser Ala Ala Asp Val Asp Asn Gly Asp
          145          150          155          160

Phe Met His Leu His Phe Ala Phe Glu Thr Thr Thr Ala Val Leu Arg
          165          170          175

Ile Val Glu Asn Phe Tyr Asp Ala Gln His Ala Thr Pro Val His Ala
          180          185          190

Leu Pro Ile Ser Ala Phe Glu Leu Lys Leu Phe Asp Asp Trp Arg Gln
          195          200          205

Trp Pro Glu Val Glu Ser Leu Ala Leu Ala Gly Ala Trp Phe Gly Ala
          210          215          220

Gly Ile Asp Phe Thr Val Asp Arg Tyr Phe Gly Pro Leu Gly Met Leu
          225          230          235          240

Ser Arg Ala Leu Gly Leu Asn Met Ser Gln Met Asn Leu His Phe Asp
          245          250          255

Gly Tyr Pro Gly Gly Cys Val Met Thr Val Ala Leu Asp Gly Asp Val
          260          265          270

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Lys Tyr Lys Leu Leu Gln Cys Val Thr Pro Val Ser Glu Gly Lys Asn
 275 280 285
 Val Met His Met Leu Ile Ser Ile Lys Lys Val Gly Gly Ile Leu Leu
 290 295 300
 Arg Ala Thr Asp Phe Val Leu Phe Gly Leu Gln Thr Arg Gln Ala Ala
 305 310 315 320
 Gly Tyr Asp Val Lys Ile Trp Asn Gly Met Lys Pro Asp Gly Gly Gly
 325 330 335
 Ala Tyr Ser Lys Tyr Asp Lys Leu Val Leu Lys Tyr Arg Ala Phe Tyr
 340 345 350
 Arg Gly Trp Val Asp Arg Val Ala Ser Glu Arg
 355 360

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28958 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CGATCGGTC GGCCTGACA CGTGAAGA GTCAAGCTC GAAGCTCCC TCGCTCTCC	60
CTCTCAAGGC ACCATTCTCA TCCAGATCTC CGTGGAGCC ATGGAAGAGG CGGACGAAG	120
GTCGCTCTCC CTCCATGGCC GGACGAGGA CGCTCTCAG GACGCCCCCTT GGACGGCCA	180
CGGAGGGGG TCGCTGCTA AAGCTGCCCC CTCCCTCTCC TTGATCTTC ACGAATGGGC	240
TCCTCGGGG GGCAAGCCGG TGGACACCCA AGGCTCTTAC GCAGGCTCG AAAGCGGGG	300
GCTCGCTAT GGGCCTCAGT TCCAGGGACT TCGCTCGTC TGGAGGCGG GCGACGAGCT	360
CTTCGCGAG GCCAAGCTCC CGGACGCAGG CGCCAAGGAT GCGCTGGT TCGCCTCCA	420
CCCCGCCCTG TTGACAGCG CCTGCAAGC GCTTGCTCTT GAAGAAGAGC GGAAGCGGG	480
CGTGGCTCTG CCTTCTCGT GGAGAGGAGT CTGGCTGCGC TCGTGGGG CCACCACTT	540
GCGGTGCGC TTCCATGTC CGAATGGCAA GTCTCGTG TCGCTCTCC TGGGAGCGC	600

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CGCAGGOGAG CCCCTOGCCT CGGTCCAAGC GCTCGCCACG CGCATCAOGT CCCAGGAGCA	660
GCTCCGCACC CAGGGAGCTT COCTCCAAGA TGCTCTCTTC CGGGTTGTCT GGAGAGATCT	720
GCCCAGCCCT AOGTCGCTCT CTGAGGCCCC GAAGGGTGTC CTCCTAGAGA CAGGGGGTCT	780
CGACCTOGOG CTGCAGGOGT CTCTGCCCG CTACGACGGT CTGCTGCCC TCGGAGGCG	840
GCTCGACCAA GGCGCTTGGC CTCGGGGCCT CGTGTGTGTC CCGTTCATCG ATTGGCCCTC	900
TGGGACCTC ATAGAGAGOG CTCACAATC CACCGCGGC GCGCTOGCCT TGCTGCAAGC	960
GTGGCTTGAC GACGAACGCC TCGCTCTCTC GCGCTCGTC CTGCTCACC GACAGGCCAT	1020
CGCAACCCAC CCGACGAGG AOGTCTGA CCTCCCTCAC GCTCTCTCT GGGGCTTGT	1080
GCGCACGCG CAAAGCGAAC ACCCGGAGCT CCGTCTCTTC CTGCTGACC TGGACCTGG	1140
TCAGGCTCG GAGGCGGCC TGCTCGGCGC GCTGACACA GGAGAGGTC AGCTGCTCT	1200
CGCCCATGGA AAATGCTCG TCCGAGGTT GGTGAATGCA CGCTGACAG AGGCGCTCAT	1260
CGCGCCGAAC GTATCCAGT GGAGCCTTCA TATCCGACC AAAGGCACCT TCGACTGCT	1320
CGCCCTCGTC GACGCTCTC TAGCCCGTGC GCGCTGCA CAAGGCCAAG TCCGCGTGC	1380
CGTGACGCG GCAGGTCTCA ACTTCGCGA TGCTCTAAC ACCCTTGGCA TGCTTCGGA	1440
CAACGCGGG CGCTCGGCG GCGAAGGCG GGGCATTGTC ACGAAGTGG GCGAGGTGT	1500
TTCCCGATAC ACTGTAGGCG ACCGGGTGAT GGGCATCTTC CGGGAGGCT TTGGCCOCAC	1560
GGTCGTGCG GACGCGCGA TGATCTGCC CATCCCGAT GCGTGGTCT TGTCCAAGC	1620
CGCCAGGTC CCGTGTCT TTCTACGCG CTACTATGGA CTGCTGATG TCGGGCATCT	1680
CAAGCCCAAT CAAGTGTCC TCATCCATGC GGCGCAGGC GGCGTCGGTA CTGCGCGCT	1740
CCAGCTGCG CGCCACCTG GCGCGAAGT CTTGCCACC GCGAGTCCAG GGAAGTGGGA	1800
CGCTCTGCG GCGCTCGGCT TCGACGATGC GCACCTGCG TCTCAAGTG ACCTGGAATT	1860
CGAGCAGCAT TTCTGCGCT CCACAGAGG GCGGGCATG GATGTGCTC TCAAGCGCTT	1920
GGCGCGGAG TTGCTGACG CTTGCTGCG TCTCTGCG AGGGTGGAA GCTTTGTGA	1980
GATGGGCAAG ACGGATATC GCGAGCCGA CGCGTAGGC CTGCGCTACC CCGCGTGT	2040
TTACCGCGC TTGATCTCT TGGAGGCTGG ACGGATGA ATTCAAGAGA TGCTGCAGA	2100
GCTGCTGAC CTGTTGAGC GCGGCTGCT TGTGCGCG CCGATCAOGT CCGGGACAT	2160
CGGCATGCG CCGAGGCGT TCGCGCGCT CGCTCAGGCG CGGCATATTG GAAAGTCTG	2220

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CCTCACCGTT	CCCGTCCCAT	CGATCCCCGA	AGGCACCATC	CTCGTCACGG	GAGGCACCGG	2280
CACGCTGGGC	GCGCTCATOG	CGCGCCACCT	CGTCGCAAT	CGGGGOGACA	AGCACCTGCT	2340
CCTCACCTOG	OGAAAGGGTG	CGAGCGCTCC	GGGGGCGAG	GCATTGOGGA	GCGAGCTCGA	2400
AGCTCTGGGG	GCTGCGGTCA	CGCTCGCCCG	GTGCGACGCG	GCGATCCAC	GCGCGCTCCA	2460
AGCCCTCTTG	GACAGCATCC	CGAGCGCTCA	CCCGCTCACG	GCGTCGTGC	ACGCGCGCGG	2520
CGCCCTTGAC	GATGGGCTGA	TCAGCGACAT	GAGCCCCGAG	CGCATCGACC	GCGTCTTTGC	2580
TCCCAAGCTC	GACGCGGCTT	GGCACTTGCA	TCAGCTCACC	CAGGACAAGG	CGCTCGGGG	2640
CTTCGTCTTC	TTCTCGTCCG	CCTCGGCGT	CCTCGGCGGT	ATGGGTCAAT	CCAACTACGC	2700
GGGGGGCAAT	GCGTTCCTTG	ACGCGCTCGC	GCATCACCGA	CGGTCCATG	GGCTCCAGG	2760
CTCTCGCTC	GCATGGGGCC	ATTGGGCGGA	GCGCAGCGGA	ATGACCGGAC	AACCTCAGCG	2820
GCGTCGATAC	CGCTCGCATG	AGGCGCGCGG	TCTCGATCC	ATCGCTCGG	ACGAGGGTCT	2880
CGCCCTCTTC	GATATGGGCG	TCGGGCGCCC	GGAGCCCGCG	CTGGTCCCG	CCCGCTTOGA	2940
CATGAACGCG	CTCGGCGCGA	AGGCGGACGG	GCTACCCCTCG	ATGTTCCAGG	GTCTCGTCCG	3000
CGCTCGGTC	GCGCGCAAGG	TCGCCAGCAA	TAATGOCCTG	GOOGGTGCG	TCACCCAGCG	3060
CCTCGCCTCC	CTCCCGCCCA	CGACCGCGA	GCGCATGCTG	CTCGATCTCG	TCGGGCGCGA	3120
AGCGGCCATC	GTCTCGGCC	TCGCCTCGTT	CGAATCGCTC	GATCCCGCTC	GCCCTCTTCA	3180
AGAGCTCGGT	CTCGATTCCC	TCATGGCCAT	CGAGCTCGGA	AATCGACTCG	CGCGCGCCAC	3240
AGGCTTGCGA	CTCCAAGCCA	CCCTCTCTTT	CGACCCCGG	ACGCGCGCGG	CGCTCGCGAC	3300
CCTGCTGCTC	GGGAAGCTCC	TCCAGCATGA	AGCTGCGGAT	CCTCGCCCT	TGGCGCGAGA	3360
GCTCGACAGG	CTAGAGGCCA	CTCTCTCGCG	GATAGCGGTG	GACGCTCAAG	CACGCGCGAA	3420
GATCATATTA	CGCTGCAAT	CCTGGTTGTC	GAAGTGAGC	GACGCTCAGG	CTGCGGACGC	3480
TGGACCGATT	CTCGGCAAGG	ATTTCAAGTC	TGCTACGAAG	GAAGAGCTCT	TCGCTGCTTG	3540
TGACGAAGCG	TTCGGAGGCC	TGGGTAAATG	AATAACGACG	AGAAGCTTGT	CTCCTACCTA	3600
CAGCAGGCGA	TGAATGAGCT	TCAGCGTGCT	CATCAGCCCC	TCGGGCGGGT	CGAAGAGAAG	3660
GAGCACGAGC	CCATCGCCAT	CGTGGCGATG	AGCTGCGGCT	TCGGGCGCGA	CGTGGCGACG	3720
CCCGAGGATC	TCTGGAAGCT	CTTGCTCGAT	GGGAAAGATG	CTATCTCGGA	CCTTCCCCCA	3780
AACCGTGGTT	GGAAGCTCGA	CGCGCTCGAC	GTCCACGGTC	GCTCCCGAGT	CGAGAGGGGA	3840
GGCTTCTTCT	ACGACGCAGA	CGCTTCGAT	CGGCGCTTCT	TCGGGATCAG	CCCACGCGAG	3900

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GCGCTCGCCA	TOGATCCCCA	GCAGGGGCTC	CTCCTCGAGA	TCTCATGGGA	AGCCTTCGAG	3960
CGTGGGGGCA	TOGACCTTGC	CTCGCTCCAA	GGGAGCCAAA	GCGGGGTCTT	CGTOGGCGTG	4020
ATACACAACG	ACTACGACGC	ATTGCTGGAG	AACGCAGCTG	GCGAACACAA	AGGATTGCTT	4080
TCCACCGGCA	GCACAGCGAG	CGTCGGCTCC	GGCGGATCG	CGTATACATT	CGGCTTTCAA	4140
GGGCCCCGCA	TCAGCGTGA	CACGGGTGC	AGCTCCTCGC	TCGTCGGGT	TCACCTCGCC	4200
TGCCAGGCC	TGCGCGTGG	CGAATGCTCC	CTGGGCTCG	CGGGGGCGT	GACGTCATG	4260
GCCACGCCAG	CAGTCTTCGT	CGGTTTCGAT	TCCGAGAGCG	CGGGGCCCC	CGATGGTTCG	4320
TGCAAGTCGT	TCTCGGTGA	GGCCAAAGGT	TGGGGCTGGG	CGAGGGGCG	CGGGATGCTC	4380
CTGCTCGAGC	GCCTCTCGA	TGCCGTCCAA	AACGGTCATC	CGTCTCTCG	CGTCTCTCGA	4440
GGCTCCGCCG	TCAACCAGGA	CGGCGGAGC	CAAGGCCTCA	CGCGGCCAA	TGGCCCTGCC	4500
CAAGAGCGCG	TCATCCGGCA	AGCGCTCGAC	AGCGCGGGC	TCACTCCAAA	GGACGTCGAC	4560
GTCGTCGAGG	CTCACGGCAC	GGGAACCACC	CTCGGAGACC	CCATCGAGGC	ACAGGCCATT	4620
CTTGCCACCT	ATGGCGAGGC	CCATTCCCAA	GACAGACCCC	TCTGGCTTGG	AAGTCTCAAG	4680
TCCAACCTGG	GACATGCTCA	GGCGCGGGC	GGGTGGGAA	GCGTCATCAA	GATGGTGCTC	4740
GCGTTGCAGC	AAGGCCTCTT	GCCCAAGACC	CTCCATGCCC	AGAATCCCTC	CCCCACATC	4800
GACTGGTCTC	CGGGCACGGT	AAAGCTCCTG	AACGAGCCCG	TCGTCTGGAC	GACCAACGGG	4860
CATCCTCGCC	ACGCGGGGT	CTCGGCTTC	GGCATCTCG	GCACCAACGC	CCAGTTCATC	4920
CTCGAAGAGG	CCCCCGCCAT	CGCGGGGTC	GAGCCGCGAG	CGTCACAGCC	CGGTCGAG	4980
CGGCTTCCCG	CAGCGTGGCC	CGTCTCTCTG	TCGGCCAAGA	GCGAGGGGC	CGTGGCGGCC	5040
CAGGCAAAGC	GGCTCCGGA	CCACCTCTC	GCCAAAAGCG	AGCTCGCCCT	CGCGATGTG	5100
GCCTATTGCG	TCGCGACCAC	GCGCGCCAC	TTCGAGCAGC	GCGCGCTCT	CTCGTCAAA	5160
GGCGCGACG	AGCTCTCTC	CGCCCTCGAT	GCGTGGCCC	AAGGACATTC	CGCGCGGTG	5220
CTCGAAGAA	GCGGGGCCCC	AGGAAAGCTC	GCGTCTCT	TCACGGGGCA	AGGAAGCCAG	5280
CGGCCCACCA	TGGGCGGGG	CCTCTACGAC	GTTTTCCCG	TCTTCGGGA	CGCCCTCGAC	5340
ACGTCGGCG	CCCACCTCGA	CGCGAGCTC	GACCGCCCC	TGCGCGACGT	CCTCTTCGCT	5400
CCGACGGCT	CGGAGCAGG	CGCGGCTC	GAGCAAACG	CCTTCACCCA	GCGGCGCTG	5460
TTTGCCCTCG	AAGTCGCCCT	CTTTCAGCTT	CTACAATCT	TCGGTCTGAA	GCCGCTCTC	5520

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CTCCTCGGAC	ACTCCATTGG	CGAGCTCGTC	GOOGCCCAOG	TOGCGGGGT	CCTTTCTCTC	5580
CAGGACGGCT	GCACCTCGT	CGCGCCCGC	GCAAAGCTCA	TGCAAGCGCT	CCCACAAGGC	5640
GGCGCCATGG	TCACCTCGG	AGCCTCGAG	GAGGAAGTCC	GCGACCTTCT	CCAGCCCTAC	5700
GAAGGCCGAG	CTAGCCTCGC	CGCCCTCAAT	GGGCTCTCT	CCACGTCGT	CGCTGGGAT	5760
GAAGACGGG	TGGTGGAGAT	CGCCCGCCAG	GOOGAAGCC	TOGGACGAA	GACCACAAGC	5820
CTGCGGTCA	GCCACGCTT	CCATTCCCG	CACATGGAG	GAATGCTGA	CGACTTCGCG	5880
CGCGTCGCCC	AGAGCCTCAC	CTACCATCCC	GCAOGCATCC	CCATCATCTC	CAACGTCACC	5940
GGCGCGCGCG	CCACGGACCA	CGAGCTCGCC	TOGCGCGACT	ACTGGGTCCG	CCACGTTGCG	6000
CACACGTC	GCCTTCCTGA	CGGCTAAGT	GOCTTCACG	CGAAGGGGC	ACGTGTCTTT	6060
CTCGAGCTCG	GGCCTCAAGC	TGTCTCTCC	GOCTTGCGC	AAGACGCCCT	CGGACAGGAC	6120
GAAGGCACT	CGCATGCGC	CTTCTTCCC	ACCTCCGCA	AGGGACGCGA	CGAAGCCGAG	6180
GCGTTCACCG	CGCGCTCGG	CGCTCTCCAC	TCCGAGGCA	TCACACCGA	CTGGAGCGCT	6240
TTCTTCGCCC	CCTTCGCTCC	ACGCAAGGTC	TCCCTCCCCA	CCTATGCTTT	CCAGCGGAG	6300
CGCTTCTGGC	CGAAGGCTC	CAAGGCACCC	GGCGCGGAG	TCAGCCACCT	TGCTCCGCTC	6360
GAGGGGGGGC	TCTGGCAAGC	CATCGAGCGC	GGGGACCTCG	ATGGGCTCAG	CGGTCAGCTC	6420
CACGTGGAG	GGAAGAGCG	GCGCGCGCG	CTCGCCCTGC	TCCTTCCAC	CCTCTCGAGC	6480
TTTCGCCACG	AGCGGCAAGA	GCAGAGCACG	GTCAGCGCT	GGCGCTACCG	TATCACCTGG	6540
AAGCCTCTGA	CCACCGCGA	AACACCGCGC	GACCTCGCG	GCACTGGCT	CGTGTGTG	6600
CGGCGGCTC	TGGACGAG	CGCGCTCCC	TCCGGGCTCA	CCGAGGCGCT	CACCGGGCGC	6660
GGCGCGCGCG	TCTCGCCTT	GCGCTGAGC	CAGGCCACCC	TGGACGCGA	GGCTCTCGCC	6720
GAGCATCTGC	GCCAGGCTTG	CGCGAGACC	GCCCCGATTC	GCGGGGTGCT	CTCGCTCTC	6780
GOCTCGAG	AGCGCCCCCT	CGCAGACCT	CCTGCCCTGC	CGCGCGGACT	CGCCCTCTCG	6840
CTTCTCTCTG	CTCAAGCCCT	CGCGACCTC	GACCTCGAGG	CGCCCTTG	GTTCTTCACG	6900
CGCGCGCGCG	TCTCCATTGG	AACTCTGAC	CCCTCGGCC	ATCCCGCCCA	GGCATGACC	6960
TGGGGCTTGG	GCGGTTCAT	CGGCTCGAG	CACCCGACC	GGTGGGGAGG	TCTGTGAGC	7020
GTCTGGGCTG	GGTTCGAG	GAGCGCGGTG	GGCGCTTGC	TGCGGGCCCT	CGCGAGCGC	7080
CACGACGAAG	ACCAGCTCGC	TCTCGCGCG	GCGGACTCT	ACGCTCGCG	CATGTGCGC	7140
GCCCCGCTCG	GCGATGCGC	TCCCGCGCG	GACTTCACGC	CGGAGGCAC	CATTCTCATC	7200

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ACCGGCGGCA CCGGOGCCAT TGGOGCTCAC GTGCGCCGAT GGCTCGCTCG AAGAGGOGCT	7260
CAGCACTCTG TCCTCATCAG CGGCGAGGC GCGAGGCCCT CTGGGCGCTC GGAGCTCCAC	7320
GACGAGCTCT CGGCCCTCGG CGGCGGCACC ACCCTGCGCG CGTGGATGT GCGGACGG	7380
AATGCTGTG CCAAGCTTCT TGAGCAGCTC GACGCGAAG GGTCGCAGGT CCGGCGCGTG	7440
TTCCACGCGA GCGGCATCGA ACACCAAGCT CGCTCGAAG CCACTCTTT CAGGGATCTC	7500
GCGAGGTTG TCTCCGGCAA GGTGGAAGGT GCAAAGCACC TCACGACCT GCTCGGCTCT	7560
CGACCCCTCG ACGCTTTGT TCTCTTTTCG TCGGCGCGG CGTCTGGGG CGGCGGACAG	7620
CAAGGCGGCT ACGCGGCGC AAACGCTTC CTGAGCGCC TTGCGAGCA TCGGCGCAGC	7680
GCTGGATTGA CAGCGACGTC GGTGGCTTG GCGCGTGGG GCGGCGCGG CATGGCCACC	7740
GATCAGGGG CAGCCACCT CCAACAGCGC GGTCTGTGCG GGATGGCCCC CTGCTTGCC	7800
CTGGCGGCGC TCGGCTGGC TCTGGAGCAC GACGAGACCA CGTCACCGT CCGGACATC	7860
GACTGGGCGC GCTTTGCGC TTGTTTCAGC GCGCTCGCC CCGCGCGCT CCTGCGGAT	7920
TTGCCCCAGG CGCAGCGCGC TCTCGAGACC AGCGAAGGCG CGTCTCGGA GCATGGCCCG	7980
GCCCCGACC TCTCGACAA GCTCGGAGC CGCTCGGAGA GCGAGCAGT TGTCTGCTC	8040
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GACCCGACA AGGGCTTCT CGATCTCGGT CTGATTGCG TCATGGCGT CGAGCTTGC	8160
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TCTCTCATC GATCGGCT CTCTTGGCG GACTGCTCG CCCACGCCCT CGGCAAGAGG	8280
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GCCATGCTCG ACCAGGTCGA CCTCTTGAC CTGCTTTCT TTGGCATCAG CCCCCGGGAG	8580
GCCAAACACC TCGACCCCA GCACGCGCTG CTCTCGAAT CTGCTGGCA GGCCCTCGAA	8640
GACGCGGCA TGTCCCCC CACCTCAAG GATCCCCCA CGGGGTCTT CGTCGGCATC	8700
GGCGCCAGCG AATACGATT GCGAGAGGG AGCACGGAAG ATTGCGAGC TTATGCGCTC	8760
CAAGGCACCG CCGGTCTT TGCGCGGGG CGCTTGGCT ACAAGCTCG CCTGCAAGGG	8820

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CCCGGCTCT	CGGTGACAC	CGCTGCTCC	TCCTGCTCG	TOGOCCTCCA	CCTOGCCTGC	8880
CAAGCCCTCC	GACAGGGGGA	GTGCAACCTC	GCCCTGCGCG	OGGGGCTCTC	CGTCATGGCC	8940
TCCCCGAGG	GCTTCGTCT	CCTTTCCCGC	CTGCGCGCCT	TGGGCCCCGA	CGGCGGCTCC	9000
AAGACCTTCT	CGGCCAACGC	CGAOGGCTAC	GGAOGGGAG	AAGGGTCAT	CGTCCCTTGC	9060
CTCGAGCGGC	TGGGTGAGC	CCTOGCCCGA	GGACACGGG	TCCTOGCCCT	CGTCCGGGGC	9120
ACCGCCATCA	ACCACGAGG	CGGTGAGC	GGTATCACCG	CCCCCAAGG	CACCTCCAG	9180
CAGAAGGTCC	TCGGGCGCG	GCTCCAGAC	GCCCGCATCA	CCCCGCGGA	CGTGAAGTC	9240
GTCGAGTGCC	ATGGCACCGG	CACCTCCTTG	GGAGACCCCA	TOGAGGTGCA	AGCCCTGGCC	9300
GCGTCTACG	CGACGGCAG	ACCGCTGAA	AAGCCTCTCC	TTCTGGGCGC	GCTCAAGACC	9360
AACATOGGCC	ATCTCGAGG	CGCCTCGGC	CTCGGGGGG	TOGCCAAGAT	CGTCGCCTCC	9420
CTCCGCCATG	AGCCCTTGC	CCCCACCCTC	CACACGGGCG	CGGCAATCC	CTTGATTGAT	9480
TGGGATACAC	TOGCCATCGA	CGTGTGAT	ACCCGAGGT	CTTGGGCCCC	CCACGAAGAT	9540
AGCAGTCCCC	GGGGGGGGG	CGTCTCGGC	TTGGGACTCT	CGGCACCAA	CGCCACGTC	9600
ATCCTCGAGG	AGGCTCCCGC	CGCCTGTGG	GGGAGCCCG	CCACCTCACA	GAGGGGTGG	9660
CGACCGCTCC	CGGGGGGGT	TGCGGTGCTC	CTGTGGGCA	GGAGCGAGGC	CGCGTCCGC	9720
GCCCAGGGA	AGGGGCTCG	CGACCACCTC	CTGCCCCAG	ACGACCTCGC	CCTTATCGAT	9780
GTGGCCTATT	CGCAGGCCAC	CACCGGGCC	CACCTGAGC	ACCGGGCGC	TCTCCTGGCC	9840
CGCGACCGG	ACGAGCTCT	CTCGGGCTC	GACTGCTCG	CCCAGGACAA	GCCGGCCCCG	9900
AGCACCGTTC	TCGGCGGAG	CGGAAGCCAC	GGCAAGGTGG	TCTTGTCTT	TCCTGGGCAA	9960
GGCTGCGAGT	GGGAAGGGAT	GGCCTCTCC	CTGCTGACT	CCTGGCGGGT	CTTCGGGCT	10020
CAGCTGGAAG	CATGGGAGG	CGGCTGGCT	CCTCACGTGG	AGTGGAGCCT	GCTCGCGTC	10080
CTGGCGCGG	ACGAGGGGG	CCCTCCCTC	GACCGGTGG	AAGTGTACA	GCCGGCCCTC	10140
TTTGCGGTCA	TGGTCTCCCT	GGCGGCCCTC	TGGGCTGGC	TOGGGTGGA	GCCGGCGGCC	10200
GTCGTGGGCC	ACAGCCAGGG	CGAGATGGC	GCGGCTTGG	TOGCAGGGC	TCTCTCCCTC	10260
GAGGACGGG	CGGCATGGC	CGCCTGGC	AGGAAAGGC	TCACCACCGT	CGGGGCAAC	10320
GGCGGCATGG	CGCGGTGGA	GCTGGGGCC	TCGACCTCC	AGACCTACCT	CGTCCCTGG	10380
GGCGACAGGC	TCTCCACGC	CGCGTCAAC	AGCCCCAGG	CTACCTCGT	ATCGGGGAG	10440
CCCGCGCGG	TOGACGGCT	GCTGACGTC	CTCACGGCA	CCAAGGTGTT	CGCCGCAAG	10500

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ATCCGCGTCG ACTACGCCTC CCACTCCGCC CAGATGGACG CCGTOCAAGA OGAGCTOGCC	10560
GCAGGTCTAG CCAACATCGC TCCTCGGACG TGGAGCTCC CTCTTTATTC GACCGTCAOC	10620
GGCACCAGGC TOGACGGCTC OGAGCTOGAC GGGCGTACT GGTATOGAAA CCTCCGGCAA	10680
ACCGTCCTGT TCTCGAGCGC GACCGAGCGG CTCTCGACG ATGGGCATOG CTTCTCOGTC	10740
GAGGTCAGCC CCCATCCCGT GCTCAAGCTC GCOCTCCGG AGACCTGOGA GCGCTCAOCG	10800
CTOGATCCCG TGTCTGTGG CTCCATTGGA CGAGAAGAAG GCCACCTCGC CGCCTGCTC	10860
CTCTCCTGGG CGGAGCTCTC TACCCGAGGC CTGCGGCTOG ACTGGAAGGA CTTCTTOGG	10920
CCCTAAGCTC CCGCAAGGT CTCCCTCCCC ACCTACCCCT TCCAGOGAGA GCGGTCTG	10980
CTCGAAGTCT CCACGGAOGA ACGCTTCOGA CGTCGCCTCC GCAGGCTGA CCTCGGCOGA	11040
CCAATCCCGC TGCTCGGGC CGCGTGGC TTGCGGACC GCGGTGGCTT TCTCTTTACA	11100
GGGCGGCTCT CCTCGCAGA GCACCGTGG CTGGAAGGCC ATGCGTCTT CGGCACACC	11160
ATCCTACCGG GCACCGGCTT TCTCGAGCTC GCCCTGCACG TCGCCACCG CGTGGGCTC	11220
GACACCGTGG AAGAGCTCAC GCTCGAGGCC CCTCTGGCTC TCCATCGCA GGACACCGTC	11280
CTCTCCAGA TCTCGTGG GCGGTGGAC GACGAGGAC GAAGGGGCT CTCTTTCCAT	11340
AGCGGACAAG AGGACGGCT TCAGGATGGC CCTGGACTC GCCACGCCAG CGGCTCTCTC	11400
TGCGGGGGA CCCCATCCCT CTGCGCGAT CTCCACGAGT GGCTCCCTC GAGTGCCATC	11460
CGGTGGACC TCGAAGGCT CTAAGCAACC CTGCGCAACC TGGGCTTGC CTAAGGCCCC	11520
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AGCGCCCTGC ATGCACTGGC CTTTGAGGAC GAGCAGAGAG GGACGGTGC TCTGCCCTTC	11700
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CGTCCCAAG GTGAATCCTC CGTCTGATC GTCTGGCG ACGCGCAGG TGACCTCTT	11820
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GCTTCCACC ATGATGCGCT CTTCCGCTC GACTGGAGCG AGCTCCAAAG CCCCATTCA	11940
CCGCTGCG CCGGAGCGG CGTCTTCTC GGCACAGG GCCACGATCT CGGCTGAC	12000
GCCCCGCTOG CCGCTACGC CGACCTCGCT GCCCTCGAA GCGCCCTGA CCAGGGGCT	12060
TGCGCTCCG GCTCGTGT CGCCCCCTT ATGATGAC CGGCAGGGA CCTCGTCCG	12120

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AGGCCCCACG AGGCCAOCGC GCTCGCACTC GOCCTCTTGC AAGCCTGGCT CGCCGACGAA	12180
CGCCTCGCCT CGTCGCGCCT CGTCCTCGTC ACCCGACGCG CGTCGCCAC CCACACOGAA	12240
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GAGCACCCAG AACTCCCGCT CTTCTCTGTC GACATCGACC TCAGCGAGGC CTCCCAGCAG	12360
GCCCTGCTAG GCGCGCTGCA CACAGGAGAA CGCCAGCTCG CCCTCCGCAA OGGGAAACCC	12420
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ACGTGGCGCC TCCATATTCC GAACAAAGGC AACTTCGACG CGCTCGCCCT CGTCGACGCC	12540
CCCGAGGCCCG AGGCGCCCTT CGCACACGGC CAAGTCGCA TCGCGGTGCA CGGGCAGGG	12600
CTCAACTTCC GCGATGTGCT CGACACCCCTT GGCATGTATC CGGGCGACGC GCGCGCGCTC	12660
GGAGGCGAAG GCGGGGCGAT CGTTACTGAA GTCGGTCCAG GTGTCTCCCG ATACACCGTA	12720
GGGACCGGG TGATGGGGGT CTTGCGGCGA GCGTTGGTTC CCAOGGCCAT CGCCGACGCC	12780
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CGCTCCACGC ATGGGCGCGG CATGGATGTC GTCTCGACT GTCTGGCACG CGAGTTCGTC	13140
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ATCCGTGAGC CGAOCOGAT CGGCTCGCC TACCTGGCG TCGTTTACCG CGCCTTCGAC	13260
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GCCTTCGCG CGCTCGCCA GCGCGGCGAT GTTGGGAAGT TGTCTCTAC CATTCOCGT	13440
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ATCCGAGGG ATCATCGAT CAGGCGGTC GTGCGCGCG CGGCGCCCT CGACGACGGG	13740
CGCTCGGTA GCATGAGCG CGAGCGCATC GCTCGGTCT TTGACCCCAA GCTCGATGCC	13800

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GCTTGCTACT	TGCATGAGCT	CACCCAGGAC	GAGCCGGTCG	CGGCTTCGT	CCTCTTCTCG	13860
GCCGCTCCG	GCGTCCTTGG	TGGTCCAGGT	CAGTCGAACT	AAGCCGCTGC	CAATGCCTTC	13920
CTCGATGCGC	TCGCACATCA	CCGGGCGGCC	CAAGGACTCC	CAGCCGCTTC	GCTCGCTTGG	13980
GGCTACTGGG	CGAGCGCAG	TGGGATGACC	CGGCACCTCA	GCGCCGCCGA	CGCCGCTCGC	14040
ATGAGGCGCG	CGGCGTCCG	GCCCCGCGAC	ACTGACGAGG	CGCTCTCCCT	CTTCGATGTG	14100
GCTCTCTTGC	GACCCGAGCC	CGCTCTGGTC	CCGCCCCCT	TCGACTACAA	CGTGCTCAGC	14160
ACGAGTGCCG	ACGGCGTGCC	CCCGCTGTTT	CAGCGTCTCG	TCCGCGCTCG	CATCGCGCGC	14220
AAGGCCGCCA	GCAATACTGC	CCTCGCCTCG	TGCTTTCAG	AGCACCTCTC	CTCCCTCCCG	14280
CCCGCCGAAC	GCGAGCGGT	CCTCCTCGAT	CTGTCGCA	CGAAGCCGC	CTCGCTCTC	14340
GGCTCGCCT	CGTTGGAATC	GCTCGATCC	CATCGCCCTC	TACAAGAGCT	CGGCTCGAT	14400
TCCCTCATGG	CCCTCGAGCT	CGAAATCGA	CTCGCCCGCG	CGCCGGGCT	GCGGCTCCAG	14460
GCTACTCTCC	TCTTCGACTA	TCCAACCCCG	ACTGCGCTCT	CAGCTTTTTT	CACGACGCAT	14520
CTCTTGGGG	GAACCACCCA	CGCCCCCGGC	GTACCGCTCA	CCCCGGGGGG	GAGCGAAGAC	14580
CCTATGCCA	TGTTGGCGAT	GAGCTGCCGC	TTCCCGGGCG	AAGTGCGCAC	GCCCGAGGAT	14640
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GTGATCCCC	AACAGCGCAT	TTTGCTCGAG	ATCACATGGG	AAGCCTTGA	GCGTGCAGGC	14880
ATCGACCGG	CCTCCCTCCA	AGGAAGCCAA	AGCGGGGTCT	TGTTGGCGT	ATGGCAGAGC	14940
GACTACCAAT	GCATCGCTGG	TGAACGCGAC	TGGCGAATAC	AAGGACTCGT	TGCCACGGT	15000
AGCGCAGCGC	GTCGTCGGG	CGAATCGCA	TACACGTTCG	GACTTCAAGG	GCCCGCCATC	15060
AGCGTGGAGA	CGGCGTGCAG	CTTCTCTGTC	GCGGTTTACC	TGCTCTGCA	GGCCCCCCCC	15120
CACGGCGAAT	ACTCCCTGGC	GCTCGCTGGC	GGCGTGACCA	TCATGGCAC	GCCAGOCATA	15180
TTCATCGCGT	TCGACTCGA	GAGCGCGGGT	GCCCCGACG	GTCGCTGCAA	GGCCTTCTCG	15240
CCGGAAGCCG	ACGGTTCCGG	CTGGGCGGAA	GGCGCCGGGA	TGCTCCTGCT	CGAGCGCCTC	15300
TCCGATGCCG	TCAAAACGG	TCATCCGCTC	CTCGCGCTCC	TTGAGGCTC	CGCGTCAAC	15360
CAGGACGGCC	GGAGCCAAGG	CCTCACCGCG	CCCAATGGCC	CTGCCAGGA	GCGGTCATC	15420

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CGGCAAGCGC TOGACAGCGC GGGGCTCACT CCAAAGGACG TOGACGTGCT OGAGGCTCAC	15480
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ATCGTAAAGC TCCTGAACGA GGCGTGGC TGGACGACCA GGGACATCC TOGCGCGCC	15780
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TCCATTGAC GCGACGAAG CCACCTCCC CGTCTCTTG CTCTCTGGG CGAGCTCTA	17100

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TGGCOGGGOC TCACGCCOGA GTGGAAGGCC TTCTTOGOGC CCTTOGCTCC COGCAAGGTC	17160
TCACTCCCCA CCTACGCCTT CCAGCGOGAG OGTTTCTGGC TOGAOGCCCC CAACGCACAC	17220
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GTOGACACCT GGOGCTACCG CATCACGTGG AGGCCTCTGA CCACGCGCGC CACGCCCGCC	17460
GACCTOGCCG GCACCTGGCT CCTCGTCTGT CGTCCGCGC TCGGCGACGA OGCGCTCCCT	17520
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CGCTCGCGG CCACCTCCAT GGAGGATCTC GCGAGGTTG TCTCGGCAA GGTACAAGGT	18420
GCAAGACACC TCAAGACCT GCTGGGCTCT CGACCCCTCG ACGCCTTGT TCTCTCTCG	18480
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GCGTGTGGG GCGGCGGG CATGGCTACC GGGCTCTGG CAGCCAGCT AGAGCAAGC	18660
GGTCTGTGC CGATGGCCCC CTCGCTGGC GTGGCGACG TCGCGCTGGC GCTGGAGCAC	18720

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GAOGAGACCA	CCCTCACOGT	CGCCGACATC	GACTGGGGGC	GCTTTGOGCC	TTCGTTCAGC	18780
GCOGCTCGCT	CCCGCCOGCT	CCTGOGOGAT	TTGCCOGAGG	CGCAGOGGC	TCTOGAAGCC	18840
AGOGCOGATG	CGTCTCOGA	GCAAGACGGG	GCCACAGGCC	TCCTOGACAA	GCTCOGAAAC	18900
CGCTOGGAGA	GOGAGCAGAT	CCACCTGCTC	TCCTOGCTGG	TGCGCCACGA	AGGGGCCCTC	18960
GTCCTGGGCC	ATACOGAOC	CTCCAGGTC	GACCCOCACA	AGGGCTTCAT	GGACCTCGGC	19020
CTOGATTGCG	TCATGACOGT	CGAGCTTCGT	CGGCGCTTGC	AGCAGGCCAC	CGGCATCAAG	19080
CTCCCGGCCA	CCCTOGCCTT	CGACCATCCC	TCTCCTCATC	GCGTCGCGCT	CTTCTTGCGC	19140
GACTCGCTCG	CCCACGCOCT	CGGCGOGAGG	CTCTCOGTCG	AGCGOGACGC	CGCGCGCTC	19200
COGGCGCTTC	GCTCGGOGAG	CGACGAGCCC	ATCGCCATCG	TCGGCATGGC	CCTCGCCTTG	19260
COGGGCGGCA	TOGGOGATGT	CGACGCTCTT	TGGGAGTTCC	TCGCCCAAGG	ACGOGAOGCC	19320
GTCGAGCCCA	TTCCCCATGC	CGATGGGAT	GCCGGTGCCC	TCTACGACCC	CGACCCOGAC	19380
GCCAAGGCCA	AGAGCTACGT	CGGCATGCC	GCCATGCTCG	ACCAGGTGGA	CCTCTTCGAT	19440
CCTGCCTTCT	TTGGCATCAG	CCCTCGOGAG	GCCAAATACC	TCGACCCCCA	GCACOGCCTG	19500
CTCCTOGAAT	CTGCCTGGCT	GGCCCTCGAG	GAGCGCGGCA	TCGTCCCTC	CACCTCAAG	19560
GATTCTCCCA	COGGGTCTT	CGTGGCATC	GGCGCCAGCG	AATACGCACT	GCGAAACAG	19620
AGCTCCGAAG	AGGTGGAAGC	GTATGCCCTC	CAAGGCACCG	COGGGTCTT	TGCGCGGGG	19680
CGCTTGGCCT	ACACGCTCGG	CCTGCAAGGG	CCCGCGCTCT	CGGTGACAC	CGCCTGCTCC	19740
TCCTCGCTCG	TGCGCCTCCA	CCTCGCCTGC	CAAGCCCTCC	GACAGGGGGA	GTGCAACCTC	19800
GCCCTCGCGG	CGGGGTCTC	CGTCATGGCC	TCCCCGGGC	TCTTCGTCT	CCTTTCCCGC	19860
ATGCGTGCTT	TGGGCGCGGA	TGGCGGCTCC	AAGACCTTCT	CGACCAAGC	CGAOGGCTAC	19920
GGACGCGGAG	AGGGGTCTGT	CGTCTTGGC	CTCGAGCGGC	TCGGGACGC	CCTCGCCCGA	19980
GGACACOGCG	TCCTCGCCT	CGTCGCGGC	ACCGCATGA	ACCATGACGG	CGGTGAGAC	20040
GGCATCACCG	CCCCCAATGG	CACCTCCAC	CAGAAGGTCC	TCGGGCGGC	GCTCCACGAC	20100
GCCCATATCG	GCCCTGCGGA	CGTCGACGTC	GTCGAATGCC	ATGGCACCGG	CACCTCCTTG	20160
GGAGACCCCA	TCGAGGTGCA	AGCCCTGGCC	GCGTCTACG	CGATGGCAG	ACCGCTGAA	20220
AAGCCTCTCC	TTCTGGGCGC	ACTCAAGACC	AACATTGGCC	ATCTGAGGC	CGCCTCCGGC	20280
CTCGCGGGCG	TCGCCAAGAT	CGTCGCTCC	CTCGCCATG	ACGCCCTGCC	CCCCACCTC	20340
CACACGACCC	CGCGCAATCC	CCTGATCGAG	TGGGATGCGC	TCGCCATGGA	CGTCGTGAT	20400

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GCCACGAGGG CGTGGGCCCC CCACGAAGAT GGCAGTCCCC GCGCGGCGG CGTCTCGCC	20460
TTCGGACTCT CCGGCACCAA CGCCCACGTT ATCCTCGAAG AGGCTCCCGC GATCCCGCAG	20520
GCCGAGCCCA CCGGGCACA GCTCGGCTCG CAGCCGCTTC CCGCAGCCTG GCGCGTGCTC	20580
CTGTGCGCCA GGAGOGAGOC GCGCGTGCGC GCGCAGCCCC AGAGGCTCCG CGACCACTC	20640
CTCGCCACG ACGACCTCGC CCTGGCGAT GTAGCCTACT CGCTCGCCAC CACCGGGCT	20700
ACCTTCGAGC ACGTGCOCG TCTCGTGGTC CACGACCGG AAGAGCTCCT CTCGCGCTC	20760
GATTCGCTCG CCCAGGGAAG GCGCGCCCCG AGCACGCTCG TCGAACGAAG CGGAAGCCAC	20820
GGCAAGGTCT TCTTCGTCTT TCCTGGGCAA GGCTCGCAGT GGAAGGGAT GGCCCTCTCC	20880
CTGCTCGATA CCTCGCCGGT CTTCCGGGCA CAGCTCGAAG CGTGGGAGCG CGCCCTCGCG	20940
CCCCACGTGG ACTGGTCGCT GCTCGGGTG CTCGCGGGG AGGAGGGCG GCGCGGCTC	21000
GACCGGGTCG ACGTGGTCCA GCGCGGCTG TTCTCGATGA TGGTCTCGCT GGCGGCCCTG	21060
TGGCGCTCCA TGGGCGTCGA GCGGACGCG GTGGTCGGCC ATAGCCAGGG CGAGATCGCC	21120
GCGGCCTGTG TGGCGGGCGC GCTGTGCTC GAGGACGCTG CCAAGCTGGT GGCGCTGCGC	21180
AGCGGTGCGC TCGTGGAGCT CGCGGGCCAG GGGGCCATGG CCGGGTGA GCTGCGGAG	21240
GCGGAGTCC CACGGGCGCT CCAGCGCTAT GGCGATCGC TCTCCATCGG GGCGATCAAC	21300
AGCCCTCGTT TCAAGACGAT CTCGCGGAG CCGCTGCG TCGCGGCCCT GCTCGCGAT	21360
CTGGAGTCG AGGGCGTCTT CGCCCTCAAG CTGAGTTACG ACTTCGCTC CCACTCGCG	21420
CAGGTCGAGT CGATTTCGGA CGAGCTCTC GATCTCTGT CGTGGCTCGA GCGCGCTCG	21480
ACGGCGGTCC CGTTCCTACT CACGCTGAGC GGCGCGCGA TCGACGGGAG CGAGCTCGAC	21540
GCGGCCTACT GGTACCGGAA CCTCGGCAG CCGGTCCGT TCGCAGACGC TGTCAAGGC	21600
CTCCTTGCG GAGAACATCG CTTCTTGTG GAGGTGAGCC CCAGTCTGT GCTGACCTG	21660
GCTTGCAAG AGCTCCTCGA AGCTCGGAG CGCTCGGGG CGGTGGTCG CTCTCTGTG	21720
AGCGACGAAG GGGATCTACG GCGCTTCCTC GTCTCGCTCT CCGAGCTCTA CGTCAACGGC	21780
TTGCCCCG ATTGGACGAC GATCCTGCG CCGGGGAGC GGTGCGCT GCGCACCTAC	21840
CCCTTCAGC GCGAGCGCTT CTGGCTCGAC GCTCCACGG CACCGCGCG CGGCTCAAC	21900
CACCTTGCTC CGCTCGAGGG GCGGTCTTG CAGGCCATCG AGAGCGGGA TATCGACCG	21960
CTCAGCGGCC AGCTCCAGT GGACGGCGAC GAGCAGCGC CGCCCTTGC CTTGCTCTT	22020

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CCCACCCCTCG CGAGCTTTTCG CCAOGAGCGG CAAGAGCAGG GCAOGGTCGA CGCCTGGGCG	22080
TACCGCATCA CGTGGAAGCC TCTGACCAAC GCCACCAAGC OCGCOGACCT GGCOGGCACC	22140
TGGCTCCTCG TCGTGCOGGC CGCTCTGGAC GACGACGCGC TCCCTTCGCG GCTCACCGAG	22200
GCGCTCGCCC GGCGGGGGCG GCGGTCTCTC GCGGTGCGCC TGAGCCAGGC CCACCTGGAC	22260
CGGAGGGCTC TCGCGAGCA CCTGGGCCAG GCTTGCGCG AGACCGGCGC GCCTCGGGCG	22320
GTGCTCTCGC TCCTCGCCCT CGACGAAAGT CCCCTCGCG ACCATGCGCG CGTGCCCGCG	22380
GGACTCGCCT TCTCGCTCAC CCTGTGCTCA GCGCTCGGCG ACATCGCCCT CGACGGGCGC	22440
TTGTGGCTCT TCACCGCGCG CGCGTCTCC GTGGGACACT CGACCCCAT CGCCCATCCG	22500
ACGCAGGCGA TGAOCTGGGG CCTGGGCGCG GTGTGCGGCC TCGAGCACCC CGAGCGCTGG	22560
GGAGGGCTCG TGAOCTGGG CGCAGCGATC GACGCGAGCG CGTGGGGCGC CTTGCTCCCG	22620
GTCTCGCCC TCGCAACGA TGAGGACCAG CTGCTCTCC GCGCGCGCG GTTCTACGCT	22680
CGCGCCTCG TCGCGCTCC GCTCGGCGAC GCGCGCGCG CAOCTACCTT CAAGCCCGA	22740
GGACCCCTCC TCATCACCGG AGGCACCGGC GCGCTGGCG CTCAGTCCG CGATGGCTC	22800
GCTCGAGAAG GCGCAGAGCA CCTGTCTCTC ATCAGCGCG GAGGGGCCA GGCGAGGGC	22860
GCCTCGGAGC TCCACGCGA GCTCACGGC CTGGGCGCG GGTACCTT CGCGCGTGT	22920
GATGTGCGCG ACAGGAGCG TGTGCGCACG CTTCTCGAGC AGCTGACGC CGAAGGGTCG	22980
CAGGTCCGCG CGGTGTTCCA CGCGGGGGC ATCGGGGCGC ACGTCCGCT CGCGCCACC	23040
TCTCTCATGG AGCTCGCGA CGTTGTCTCT GCGAAGGTCC TAGGCGCAGG GAACCTCCAC	23100
GACCTGCTCG GTCCTCGACC CTOGACGCC TTGTCTTTT TCTGTCCAT CGCAGGGTC	23160
TGGGGCGGCG GACAACAAGC CGGATAAGC GCGGAAACG CTTCTCTGA CGCCTGGCC	23220
GACCAGCGGC GCAGTCTTGG ACAGCGGAC ACGTCCGTGG TGTGGGGCGC GTGGGGGGC	23280
GGCGGTGGTA TATTCAAGG GCGCTGGCA GCGAGCTGG AGCAAGTCG TCTGTGCGCG	23340
ATGGCCCTT CGCTGGCGT GGCGGGGCTC GCGCAAGCCC TGGAGCACGA CGAGACCACC	23400
GTCACGTCG CGACATCGA CTGGGCGCG TTTGCGCCTT CGATCAGGT CGCTCGCTCC	23460
CGCGCTCTT GCGGACTTG CCGAGCAGC GCGCCTCGA AGACAGAGAA GCGGCTCCT	23520
CCTCGAGCA CGGCCGGCC CCGGACCTC CTGACAAGC TCGGAGCG CTGCGAGAGC	23580
GAGCAGCTCC GTCTGCTCG CGGCTGGTG TGCGAGAGA CGGCCCTGT CCTCGGCAC	23640
GAAGGCGCT TCCAGCTCG ACCCGACAA GGCTTCTTCG ACCTGGTCT CGATTGATC	23700

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ATGACCGTGG AGCTTCGTGG GCGCTTGCAA CAGGCCACCG GCATCAAGCT CCOGGGCACC	23760
CTCGCCTTGG ACCATCCCTC TCCTCATGGC GTGGGGCTCT TCATGGCGGA CTGGCTGGCC	23820
CACGCCCTCG GCACGAGGCT CTCGGCGGAG GGGACGCGCG CGGGCTCGGG CCGCGCCTCG	23880
AGCGACGAGC CCATGGCCAT GGTGGGCATG GGCCTGGGCC TGCGGGGGGG GGTGGGGAT	23940
GTOGAGCTC TTTGGGAGTT CCTCCACCAA GGGGGCGACG CGGTGGAGCC CATTCACAG	24000
AGCGGCTGGG ACGCGGTGC CCTCTAGAC CCGACCCCG ACGCGAGCG CAAGAGCTAC	24060
GTGGGGCATG CCGGATGCT CGACGAGATC GACCTCTTCG ACCCTGCCTT CTGGGCATC	24120
AGCCCCGGG AGGCCAAACA CCTGGACCCC CAGCACGGCC TGCTCCTGA ATCTGCCTGG	24180
CTGGCCCTCG AGGACGCGG CATCGTCCCC ACCTCCCTCA AGGACTCCCT CACGGGGTC	24240
TTCGTGGCA TCTGGCGCG CGAATAAGCG ATGCAAGAGG CGAGCTCGGA AGGTTCGAG	24300
GTTTACTTCA TCCAAGGCAC TTCGGGTCC TTTGGCGGG GGGGCTTGGC CTATAGCTC	24360
GGGCTCCAGG GCGGGGATC TTCGGTGGAC ACGGCTGCT CCTCTCGCT GGTCTCCCTC	24420
CACCTGGCT GCGAAGCCCT CCGACAGGGC GAGTCAACC TGGCCCTGC CGGGGGGTG	24480
TGGCTCATGG TCTCCCCCA GACCTTCGTC ATCTTTCCG GTCTGGGGC CTGGGGGCC	24540
GACGGCGCT CCAAGACCTT CTCGGACAAC GCGACGGCT ACGGACGGG AGAAGGGTC	24600
GTCGTCTTG CCTGGAGCG GATGGGGAC GGCCTGGCC GGAGACACG GGTCTCTGTC	24660
CTGTTCGGG GCACCGCAT CAACCAAGC GGGGGTGA GGGTATCAC GGGGGCAAC	24720
GGCAGCTCC AGCAGAAGGT CCTCGGGGC GGGCTCCAG ACGGGCAT CAGGGGGGC	24780
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CAAGCCCTGG CCGCGTCTA GCGGACGGC AGACCGCTG AAAAGCTCT CTCTCTGGC	24900
GCGCTCAAGA CCAACATGG CCATCTGAG GCGGCTCG GCTCGGGG GGTGGCAAG	24960
ATGGTGGCT GGTCCGCA GACGCGCTG CCCCCACCC TCAAGGAGC CCAAGCAAT	25020
CCCTCATGG AGTGGGAGG GCTCGGCATC GAGTGGTGG ATACCGGAG GCTTGGGCC	25080
CGCCACGAAG ATGGCAGTC CCGCGGGC GGCATCTCG CCTTGGATT CTGGGGCAC	25140
AACGCCACG TCATCTGGA AGAGGCTCC GCGGCTGC GGGCGAGC GGCACCTCA	25200
CAGCGGGGT GCGAAGCGC TCCGGGGG TGGCCGTGC TCCTGTGGC CAGGAGGAG	25260
GCGCGGTCC GCGCCAGG GAAGGGCTC GCGACACC TGTGGCCA GACGACCTC	25320

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ACCCCTCGGG ATGTGGCCTA TTOGCTGGCC ACCACCCGGG COCACTTCGA GCACCGGGCC	25380
GCTCTCGTAG CCCACAACCG CGACGAGCTC CTCTCCGGGC TOGACTCGCT CGCCAGGAC	25440
AAGCCCGCCC CGAGCACCGT CCTGGGAAGG AGGGGAAGCC ACGGCAAGCT CGTCTTCGTC	25500
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GTCTTCGGG CTCAGCTOGA AGCATGCGAG CGCGCGCTCG CTCTCACGT CGAGTGGAGC	25620
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CAGCCCGCCC TCCTTGCCGT CATGGTCTCC CTGGGGCCC TCTGGGCTC GCTCGGGTA	25740
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GACGAGCTCG CGCAGGTCT AGCCAACATC GCTCTCGGA CGTGCGAGCT CCTCTTTAT	26160
TCGACGTCA CGGCACCAG GCTCGACGGC TCGAGCTCG ACGGCGGTA CTGGTATOGA	26220
AACCTCCGGC AAACCGTCT GTTCTCGAGC GCGACGAGC GGCTCCTCGA CGATGGGCAT	26280
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GAGCGCTCAC CGCTCGATCC CGTGTGTC GGCTCATTC GACGGAAGA AGGOCACCTC	26400
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ACCTCGGCG ACCACCGCT GCTCGGGCC GCGTCGCC TOGCGACCG CGATGGCTTT	26640
GTCTTCACAG GACGGCTCTC CTOGCGAG CACCGTGGC TCGAAGACCA CGTGTCTTC	26700
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TCCGTTTATA GCGGGCGCA CGACGCGCT CAGGATGGC CCTGGACTCG CCACGCCAGC	26940
GGCTCTCTCG CGCAAGCTAG CCGTCCCAT TGCTTCGAT GTCGCGGAA TGGCCCCC	27000

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TCGGGGGCCA CCCAGGTGGA CACCCAAGGT TTCTACGCAG COCTCGAGAG OGCTGGGCTT	27060
GCTTATGGCC COGAGTTOCA GGGCCTCCGC CGCGTCTAC AAGOGCGGCG AOGAGCTCTT	27120
CGCCGAAGCC AAGCTCCCGG ACGCCGCCGA AGAGGACGCC GCTCGTTTTG CCCTCCACCC	27180
CGCCCTGCTC GACAGCGCT TGCAGGCGCT CGCCTTTGTA GACGACCAGG CAAAGGCTT	27240
CAGGATGCCC TTCTCGTGGA GGGAGTATC GCTGCGCTCC GGTGGGAGCC ACCACCCTGC	27300
GCGTGGGTTT CCACCGTCTT GAGGGCGAAT CCTCGCGCTC GCTCCTCTC GCCGAGCCA	27360
GAGGCGAACC CATCGCCTCG GTGCAAGCGC TCGCCATGCG CGCCGCGTCC GCGAGCAGC	27420
TCCGCAGACC CGGGAGCGTC CCACCTOGAT GCCCTCTTCC GCATCGACTG GAGCGAGCTG	27480
CAAAGCCCCA CCTCACCGCC CATCGCCCG AGCGGTGCC TCCTCGGCAC AGAAGGTCTC	27540
GACCTCGGGA CCAGGGTGCC TCTCGACGC TATACGACC TTGCTGCTCT ACGCAGCGCC	27600
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GGCGACCTCA TCGCGAGCGC CGCGAGACC ACCGCGCAG CGCTCGCCCT CTGCAAGCC	27720
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GCCACCCACG CTGAAGAAGA CGTCAAGGGC CTGCTCAG CGCCTCTCTG GGTCTCGCT	27840
CGCTCGCGC AGAGCGAGCA CCCAGAGCGC CCTCTCGTCC TGTGACCT CGACGACAGC	27900
GAGGCCTCCC AGCACGCCCT GCTCGGCGCG CTGACGCAA GAGAGCCAGA GATCGCCCTC	27960
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GCTTCGGTCA CCTCGCGCG GTGCGACGTG GCGATCCAC GCGCTCTAAA GGACCTTCTG	28260
GATAACATTG CGAGCGCTCA CCGGTGCGC GCGTCTGTC ATGCGCCAG CGTCTCGAC	28320
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GATGCGCCT GGCATTGCA TCAGCTCAC CAAGATAAGC CCTTGCGCG CTTCATCTC	28440
TTCTCGTCCG TCGCGGCGT CCTCGGCAGC TCAGGTCACT CCAACTACGC CGCTGCGAGC	28500
GCTTCTCTG ATGCGCTGTC GCACCACGG CGCGCGCAAG GGCTCCCTGC CTCATGCTC	28560
GCGTGGAGCC ACTGGGCGA GGCAGCGCA ATGACAGAGC ACGTCAGCG CGCGGCGCC	28620

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CCTCGCATGG AGCGCGCOGG CCTTCCCTCG ACCTCTGAGG AGAGGCTCGC CCTCTTGGAT 28680
 GCGGCGCTCT TCCGAACCGA GACCGCCCTG GTCCCGGCGC GCTTCGACTT GAGGCGGCTC 28740
 AGGGCGAACG CCGGCAGOGT CCCCCGGTTG TTCCAACGTC TCGTCGGGCG TCGCACGGTA 28800
 CGCAAGGCCG CCAGCAACAC CGCCAGGOC TCGTCGCTTA CAGAGCGOCT CTCAGCCCTC 28860
 CGCCCGGCGG AACGOGAGOG TGCCCTGCTC GATCTCATCC GCACCGAAGC CGCCGCGCTC 28920
 CTCGGCCTCG CCTCCTTCGA ATCGCTCGAT CCGATCG 28958

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..13
- (D) OTHER INFORMATION: /note= "sequence of a plant consensus translation initiator (Clontech)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GTCGACCATG GTC

13

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: misc_feature

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- (B) LOCATION: 1..12
- (D) OTHER INFORMATION: /note= "sequence of a plant consensus translation initiator (Joshi)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TAAACAATGG CT

12

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (ix) FEATURE:
 - (A) NAME/KEY: misc_feature
 - (B) LOCATION: 1..22
 - (D) OTHER INFORMATION: /note= "sequence of an oligonucleotide for use in a molecular adaptor"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

AATTCTAAAG CATGCCGATC GG

22

(2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (ix) FEATURE:
 - (A) NAME/KEY: misc_feature
 - (B) LOCATION: 1..21
 - (D) OTHER INFORMATION: /note= "sequence of an oligonucleotide for use in a molecular adaptor"

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AATTCOGATC GGCATGCTTT A

21

(2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 22 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: misc feature
 (B) LOCATION: 1..22
 (D) OTHER INFORMATION: /note= "sequence of an
 oligonucleotide for use in a molecular adaptor"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

AATTCTAAAC CATGGCGATC GG

22

(2) INFORMATION FOR SEQ ID NO: 12:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: misc feature
 (B) LOCATION: 1..21
 (D) OTHER INFORMATION: /note= "sequence of an
 oligonucleotide for use in a molecular adaptor"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

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AATTCOGATC GCCATGGTTT A

21

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..15
- (D) OTHER INFORMATION: /note= "sequence of an
oligonucleotide for use in a molecular adaptor"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CCAGCTGGAA TTCCG

15

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..19
- (D) OTHER INFORMATION: /note= "sequence of an
oligonucleotide for use in a molecular adaptor"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CGGAATTCCA GCTGGCATG

19

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(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..11
- (D) OTHER INFORMATION: /note= "oligonucleotide used to introduce base change into SphI site of ORF1 of pyrrolnitrin gene cluster"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CCCCCTCATG C

11

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..11
- (D) OTHER INFORMATION: /note= "oligonucleotide used to introduce base change into SphI site of ORF1 of pyrrolnitrin gene cluster"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GCATGAGGGG G

11

(2) INFORMATION FOR SEQ ID NO: 17:

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- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 4603 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 230..1597
 (D) OTHER INFORMATION: /gene= "phz1"

/label= ORF1

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 1598..2761
 (D) OTHER INFORMATION: /gene= "phz2"

/label= ORF2

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 2764..3600
 (D) OTHER INFORMATION: /gene= "phz3"

/label= ORF3

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
 (B) LOCATION: 3597..4265
 (D) OTHER INFORMATION: /label= ORF4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GCATGCOGTG ACCTCGCOG GTGGGTGGC OGOOGGCTG CACCTGGAAA CCACCCCTGA	60
CGACGTCAGC GAGTGGGCTT CGATGCOGC CGGCTGTCAT CAGGTGGCCA GCGCTACAA	120
AAGCCTGTGC GACCCGCGCC TGAACCCCTG GCAAGCCATT ACTGCGGTGA TGGCTGGAA	180
AAACCAGCCC TCTTCAACCC TTGCTCTCTT TTGACTGGAG TTTGTGTC ATG ACC	235
	Met Thr
	1
GGC ATT CCA TGG ATC GTC CCT TAC GGC TTG OCT ACC AAC CGC GAC CTG	283
Gly Ile Pro Ser Ile Val Pro Tyr Ala Leu Pro Thr Asn Arg Asp Leu	
5 10 15	
CCC GTC AAC CTC GCG CAA TGG AGC ATC GAC CCC GAG CGT GCC GTG CTG	331

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Pro	Val	Asn	Leu	Ala	Gln	Trp	Ser	Ile	Asp	Pro	Glu	Arg	Ala	Val	Leu	
20						25					30					
CTG	GTG	CAT	GAC	ATG	CAG	CGC	TAC	TTC	CTG	CGG	CCC	TTG	CCC	GAC	GCC	379
Leu	Val	His	Asp	Met	Gln	Arg	Tyr	Phe	Leu	Arg	Pro	Leu	Pro	Asp	Ala	
35					40				45						50	
CTG	CGT	GAC	GAA	GTC	GTG	AGC	AAT	GCC	GCG	CGC	ATT	CGC	CAG	TGG	GCT	427
Leu	Arg	Asp	Glu	Val	Val	Ser	Asn	Ala	Ala	Arg	Ile	Arg	Gln	Trp	Ala	
				55				60						65		
GCC	GAC	AAC	GGC	GTT	CCG	GTG	GCC	TAC	ACC	GCC	CAG	CCC	GGC	AGC	ATG	475
Ala	Asp	Asn	Gly	Val	Pro	Val	Ala	Tyr	Thr	Ala	Gln	Pro	Gly	Ser	Met	
			70					75					80			
AGC	GAG	GAG	CAA	CGC	GGG	CTG	CTC	AAG	GAC	TTC	TGG	GGC	CCG	GGC	ATG	523
Ser	Glu	Glu	Gln	Arg	Gly	Leu	Leu	Lys	Asp	Phe	Trp	Gly	Pro	Gly	Met	
	85						90					95				
AAG	GCC	AGC	CCC	GCC	GAC	CGC	GAG	GTG	GTC	GGC	GCC	CTG	ACG	CCC	AAG	571
Lys	Ala	Ser	Pro	Ala	Asp	Arg	Glu	Val	Val	Gly	Ala	Leu	Thr	Pro	Lys	
100						105					110					
CCC	GGC	GAC	TGG	CTG	CTG	ACC	AAG	TGG	CGC	TAC	AGC	GCG	TTC	TTC	AAC	619
Pro	Gly	Asp	Trp	Leu	Leu	Thr	Lys	Trp	Arg	Tyr	Ser	Ala	Phe	Phe	Asn	
115					120					125					130	
TCC	GAC	CTG	CTG	GAA	CGC	ATG	CGC	GCC	AAC	GGG	CGC	GAT	CAG	TTG	ATC	667
Ser	Asp	Leu	Leu	Glu	Arg	Met	Arg	Ala	Asn	Gly	Arg	Asp	Gln	Leu	Ile	
				135					140					145		
CTG	TGC	GGG	GTG	TAC	GCC	CAT	GTC	GGG	GTA	CTG	ATT	TCC	ACC	GTG	GAT	715
Leu	Cys	Gly	Val	Tyr	Ala	His	Val	Gly	Val	Leu	Ile	Ser	Thr	Val	Asp	
			150					155					160			
GCC	TAC	TCC	AAC	GAT	ATC	CAG	CCG	TTC	CTC	GTT	GCC	GAC	GCG	ATC	GCC	763
Ala	Tyr	Ser	Asn	Asp	Ile	Gln	Pro	Phe	Leu	Val	Ala	Asp	Ala	Ile	Ala	
		165					170					175				
GAC	TTC	AGC	AAA	GAG	CAC	CAC	TGG	ATG	CCA	TCG	AAT	ACG	CCG	CCA	GCC	811
Asp	Phe	Ser	Lys	Glu	His	His	Trp	Met	Pro	Ser	Asn	Thr	Pro	Pro	Ala	
	180					185					190					
GTT	GCG	CCA	TGT	CAT	CAC	CAC	CGA	CGA	GGT	GGT	GCT	ATG	AGC	CAG	ACC	859
Val	Ala	Pro	Cys	His	His	His	Arg	Arg	Gly	Gly	Ala	Met	Ser	Gln	Thr	
195					200				205						210	
GCA	GCC	CAC	CTC	ATG	GAA	CGC	ATC	CTG	CAA	CCG	GCT	CCC	GAG	CCG	TTT	907
Ala	Ala	His	Leu	Met	Glu	Arg	Ile	Leu	Gln	Pro	Ala	Pro	Glu	Pro	Phe	
				215				220					225			
GCC	CTG	TTG	TAC	CGC	CCG	GAA	TCC	AGT	GGC	CCC	GGC	CTG	CTG	GAC	GTG	955
Ala	Leu	Leu	Tyr	Arg	Pro	Glu	Ser	Ser	Gly	Pro	Gly	Leu	Leu	Asp	Val	
			230					235					240			

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CTG ATC GGC GAA ATG TCG GAA CCG CAG GTC CTG GGC GAT ATC GAC TTG Leu Ile Gly Glu Met Ser Glu Pro Gln Val Leu Ala Asp Ile Asp Leu 245 250 255	1003
OCT GCC ACC TCG ATC GGC GCG CCT CGC CTG GAT GTA CTG GCG CTG ATC Pro Ala Thr Ser Ile Gly Ala Pro Arg Leu Asp Val Leu Ala Leu Ile 260 265 270	1051
CCC TAC CGC CAG ATC GGC GAA CCG GGT TTC GAG GCG GTG GAC GAT GAG Pro Tyr Arg Gln Ile Ala Glu Arg Gly Phe Glu Ala Val Asp Asp Glu 275 280 285 290	1099
TCG CCG CTG CTG GCG ATG AAC ATC ACC GAG CAG CAA TCC ATC AGC ATC Ser Pro Leu Leu Ala Met Asn Ile Thr Glu Gln Gln Ser Ile Ser Ile 295 300 305	1147
GAG CGC TTG CTG GGA ATG CTG CCC AAC GTG CCG ATC CAG TTG AAC AGC Glu Arg Leu Leu Gly Met Leu Pro Asn Val Pro Ile Gln Leu Asn Ser 310 315 320	1195
GAA CGC TTC GAC CTC AGC GAC GCG AGC TAC GCC GAG ATC GTC AGC CAG Glu Arg Phe Asp Leu Ser Asp Ala Ser Tyr Ala Glu Ile Val Ser Gln 325 330 335	1243
GTG ATC GGC AAT GAA ATC GGC TCC GGG GAA GGC GCC AAC TTC GTC ATC Val Ile Ala Asn Glu Ile Gly Ser Gly Glu Gly Ala Asn Phe Val Ile 340 345 350	1291
AAA CGC ACC TTC CTG GCC GAG ATC AGC GAA TAC GGC CCG GCC AGT GCG Lys Arg Thr Phe Leu Ala Glu Ile Ser Glu Tyr Gly Pro Ala Ser Ala 355 360 365 370	1339
CTG TCG TTC TTT CGC CAT CTG CTG GAA CCG GAG AAA GGC GCC TAC TGG Leu Ser Phe Phe Arg His Leu Leu Glu Arg Glu Lys Gly Ala Tyr Trp 375 380 385	1387
ACG TTC ATC ATC CAC ACC GGC AGC CGT ACC TTC GTG GGT GCG TCC CCC Thr Phe Ile Ile His Thr Gly Ser Arg Thr Phe Val Gly Ala Ser Pro 390 395 400	1435
GAG CGC CAC ATC AGC ATC AAG GAT GGG CTC TCG GTG ATG AAC CCC ATC Glu Arg His Ile Ser Ile Lys Asp Gly Leu Ser Val Met Asn Pro Ile 405 410 415	1483
AGC GGC ACT TAC CGC TAT CCG CCC GCC GGC CCC AAC CTG TCG GAA GTC Ser Gly Thr Tyr Arg Tyr Pro Pro Ala Gly Pro Asn Leu Ser Glu Val 420 425 430	1531
ATG GAC TTC CTG GCG GAT CGC AAG GAA GCC GAC GAG CTC TAC ATG GTG Met Asp Phe Leu Ala Asp Arg Lys Glu Ala Asp Glu Leu Tyr Met Val 435 440 445 450	1579
GTG GAT GAA GAG CTG TAA ATG ATG GCG CGC ATT TGT GAG GAC GGC GGC Val Asp Glu Glu Leu * Met Met Ala Arg Ile Cys Glu Asp Gly Gly 455 1 5 10	1627

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CAC GTC CTC GGC OCT TAC CTC AAG GAA ATG GCG CAC CTG GCC CAC ACC His Val Leu Gly Pro Tyr Leu Lys Glu Met Ala His Leu Ala His Thr 15 20 25	1675
GAG TAC TTC ATC GAA GGC AAG ACC CAT CGC GAT GTA CCG GAA ATC CTG Glu Tyr Phe Ile Glu Gly Lys Thr His Arg Asp Val Arg Glu Ile Leu 30 35 40	1723
CGC GAA ACC CTG TTT GCG CCC ACC GTC ACC GGC AGC CCA CTG GAA AGC Arg Glu Thr Leu Phe Ala Pro Thr Val Thr Gly Ser Pro Leu Glu Ser 45 50 55	1771
GCC TGC CCG GTC ATC CAG CGC TAT GAN CCG CAA GGC CGC GCG TAC TAC Ala Cys Arg Val Ile Gln Arg Tyr Xaa Pro Gln Gly Arg Ala Tyr Tyr 60 65 70	1819
AGC GGC ATG GCT GCG CTG ATC GGC AGC GAT GGC AAG GGC GGG CGT TCC Ser Gly Met Ala Ala Leu Ile Gly Ser Asp Gly Lys Gly Gly Arg Ser 75 80 85 90	1867
CTG GAC TCC GCG ATC CTG ATT CGT ACC GCC GAC ATC GAT AAC AGC GGC Leu Asp Ser Ala Ile Leu Ile Arg Thr Ala Asp Ile Asp Asn Ser Gly 95 100 105	1915
GAG GTG CCG ATC AGC GTG GGC TCG ACC ATC GTG CCG CAT TCC GAC CCG Glu Val Arg Ile Ser Val Gly Ser Thr Ile Val Arg His Ser Asp Pro 110 115 120	1963
ATG ACC GAG GCT GCC GAA AGC CCG GCC AAG GCC ACT GGC CTG ATC AGC Met Thr Glu Ala Ala Glu Ser Arg Ala Lys Ala Thr Gly Leu Ile Ser 125 130 135	2011
GCA CTG AAA AAC CAG GCG CCC TCG CGC TTC GGC AAT CAC CTG CAA GTG Ala Leu Lys Asn Gln Ala Pro Ser Arg Phe Gly Asn His Leu Gln Val 140 145 150	2059
CGC GCC GCA TTG GCC AGC CGC AAT GCC TAC GTC TOG GAC TTC TGG CTG Arg Ala Ala Leu Ala Ser Arg Asn Ala Tyr Val Ser Asp Phe Trp Leu 155 160 165 170	2107
ATG GAC AGC CAG CAG CCG GAG CAG ATC CAG GCC GAC TTC AGT GGG CGC Met Asp Ser Gln Gln Arg Glu Gln Ile Gln Ala Asp Phe Ser Gly Arg 175 180 185	2155
CAG GTG CTG ATC GTC GAC GCC GAA GAC ACC TTC ACC TCG ATG ATC GCC Gln Val Leu Ile Val Asp Ala Glu Asp Thr Phe Thr Ser Met Ile Ala 190 195 200	2203
AAG CAA CTG CCG GCC CTG GGC CTG GTA GTG ACG GTG TGC AGC TTC AGC Lys Gln Leu Arg Ala Leu Gly Leu Val Val Thr Val Cys Ser Phe Ser 205 210 215	2251
GAC GAA TAC AGC TTT GAA GGC TAC GAC CTG GTC ATC ATG GGC CCC GGC Asp Glu Tyr Ser Phe Glu Gly Tyr Asp Leu Val Ile Met Gly Pro Gly	2299

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220	225	230	
CCC GGC AAC CCG AGC GAA GTC CAA CAG CCG AAA ATC AAC CAC CTG CAC Pro Gly Asn Pro Ser Glu Val Gln Gln Pro Lys Ile Asn His Leu His 235 240 245 250			2347
GTG GCC ATC CGC TCC TTG CTC AGC CAG CAG CCG CCA TTC CTC GCG GTG Val Ala Ile Arg Ser Leu Leu Ser Gln Gln Arg Pro Phe Leu Ala Val 255 260 265			2395
TGC CTG AGC CAT CAG GTG CTG AGC CTG TGC CTG GGC CTG GAA CTG CAG Cys Leu Ser His Gln Val Leu Ser Leu Cys Leu Gly Leu Glu Leu Gln 270 275 280			2443
CGC AAA GCC ATT CCC AAC CAG GGC GTG CAA AAA CAG ATC GAC CTG TTT Arg Lys Ala Ile Pro Asn Gln Gly Val Gln Lys Gln Ile Asp Leu Phe 285 290 295			2491
GGC AAT GTC GAA CCG GTG GGT TTC TAC AAC ACC TTC GCC GCC CAG AGC Gly Asn Val Glu Arg Val Gly Phe Tyr Asn Thr Phe Ala Ala Gln Ser 300 305 310			2539
TCG AGT GAC CGC CTG GAC ATC GAC GGC ATC GGC ACC GTC GAA ATC AGC Ser Ser Asp Arg Leu Asp Ile Asp Gly Ile Gly Thr Val Glu Ile Ser 315 320 325 330			2587
CGC GAC AGC GAG ACC GGC GAG GTG CAT GCC CTG CGT GGC CCC TCG TTC Arg Asp Ser Glu Thr Gly Glu Val His Ala Leu Arg Gly Pro Ser Phe 335 340 345			2635
GCC TCC ATG CAG TTT CAT GCC GAG TCG CTG CTG ACC CAG GAA GGT CCG Ala Ser Met Gln Phe His Ala Glu Ser Leu Leu Thr Gln Glu Gly Pro 350 355 360			2683
CGC ATC ATC GCC GAC CTG CTG CGG CAC GCC CTG ATC CAC ACA CCT GTC Arg Ile Ile Ala Asp Leu Leu Arg His Ala Leu Ile His Thr Pro Val 365 370 375			2731
GAG AAC AAC GCT TCG GCC GCC GGG AGA TAA CC ATG CAC CAT TAC GTC Glu Asn Asn Ala Ser Ala Ala Gly Arg * Met His His Tyr Val 380 385 1 5			2778
ATC ATC GAC GCC TTT GCC AGC GTC CCG CTG GAA GGC AAT CCG GTC GCG Ile Ile Asp Ala Phe Ala Ser Val Pro Leu Glu Gly Asn Pro Val Ala 10 15 20			2826
GTG TTC TTT GAC GCC GAT GAC TTG TCG GCC GAG CAA ATG CAA CGC ATT Val Phe Phe Asp Ala Asp Asp Leu Ser Ala Glu Gln Met Gln Arg Ile 25 30 35			2874
GCC CCG GAG ATG AAC CTG TCG GAA ACC ACT TTC GTG CTC AAG CCA CGT Ala Arg Glu Met Asn Leu Ser Glu Thr Thr Phe Val Leu Lys Pro Arg 40 45 50			2922
AAC TGC GGC GAT GCG CTG ATC CGG ATC TTC ACC CCG GTC AAC GAA CTG			2970

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Asn	Cys	Gly	Asp	Ala	Leu	Ile	Arg	Ile	Phe	Thr	Pro	Val	Asn	Glu	Leu	
55						60					65					
CCC	TTC	GCC	GGG	CAC	CCG	TTG	CTG	GGC	ACG	GAC	ATT	GCC	CTG	GGT	GCG	3018
Pro	Phe	Ala	Gly	His	Pro	Leu	Leu	Gly	Thr	Asp	Ile	Ala	Leu	Gly	Ala	
70					75				80					85		
CGC	ACC	GAC	AAT	CAC	CGG	CTG	TTC	CTG	GAA	ACC	CAG	ATG	GGC	ACC	ATC	3066
Arg	Thr	Asp	Asn	His	Arg	Leu	Phe	Leu	Glu	Thr	Gln	Met	Gly	Thr	Ile	
				90					95					100		
GCC	TTT	GAG	CTG	GAG	CGC	CAG	AAC	GGC	AGC	GTC	ATC	GCC	GCC	AGC	ATG	3114
Ala	Phe	Glu	Leu	Glu	Arg	Gln	Asn	Gly	Ser	Val	Ile	Ala	Ala	Ser	Met	
			105					110					115			
GAC	CAG	CCG	ATA	CCG	ACC	TGG	ACG	GCC	CTG	GGG	CGC	GAC	GCC	GAG	TTG	3162
Asp	Gln	Pro	Ile	Pro	Thr	Trp	Thr	Ala	Leu	Gly	Arg	Asp	Ala	Glu	Leu	
		120					125					130				
CTC	AAG	GCC	CTG	GGC	ATC	AGC	GAC	TCG	ACC	TTT	CCC	ATC	GAG	ATC	TAT	3210
Leu	Lys	Ala	Leu	Gly	Ile	Ser	Asp	Ser	Thr	Phe	Pro	Ile	Glu	Ile	Tyr	
	135					140					145					
CAC	AAC	GGC	CCG	CGT	CAT	GTG	TTT	GTC	GGC	CTG	CCA	AGC	ATC	GCC	GCG	3258
His	Asn	Gly	Pro	Arg	His	Val	Phe	Val	Gly	Leu	Pro	Ser	Ile	Ala	Ala	
150					155					160					165	
CTG	TCG	GCC	CTG	CAC	CCC	GAC	CAC	CGT	GCC	CTG	TAC	AGC	TTC	CAC	GAC	3306
Leu	Ser	Ala	Leu	His	Pro	Asp	His	Arg	Ala	Leu	Tyr	Ser	Phe	His	Asp	
				170					175					180		
ATG	GCC	ATC	AAC	TGT	TTT	GCC	GGT	GCG	GGA	CGG	CGC	TGG	CGC	AGC	CGG	3354
Met	Ala	Ile	Asn	Cys	Phe	Ala	Gly	Ala	Gly	Arg	Arg	Trp	Arg	Ser	Arg	
			185					190					195			
ATG	TTC	TCG	CCG	GCC	TAT	GGG	GTG	GTC	GAG	GAT	GCG	NCC	ACG	GGC	TCC	3402
Met	Phe	Ser	Pro	Ala	Tyr	Gly	Val	Val	Glu	Asp	Ala	Xaa	Thr	Gly	Ser	
		200					205					210				
GCT	GCC	GGG	CCC	TTG	GCG	ATC	CAT	CTG	GCG	CGG	CAT	GGC	CAG	ATC	GAG	3450
Ala	Ala	Gly	Pro	Leu	Ala	Ile	His	Leu	Ala	Arg	His	Gly	Gln	Ile	Glu	
		215				220					225					
TTC	GGC	CAG	CAG	ATC	GAA	ATT	CTT	CAG	GGC	GTG	GAA	ATC	GGC	CGC	CCC	3498
Phe	Gly	Gln	Gln	Ile	Glu	Ile	Leu	Gln	Gly	Val	Glu	Ile	Gly	Arg	Pro	
230					235					240					245	
TCA	CTC	ATG	TTC	GCC	CGG	GCC	GAG	GGC	CGC	GCC	GAT	CAA	CTG	ACG	CGG	3546
Ser	Leu	Met	Phe	Ala	Arg	Ala	Glu	Gly	Arg	Ala	Asp	Gln	Leu	Thr	Arg	
				250					255					260		
GTC	GAA	GTA	TCA	GGC	AAT	GGC	ATC	ACC	TTC	GGA	CGG	GGG	ACC	ATC	GTT	3594
Val	Glu	Val	Ser	Gly	Asn	Gly	Ile	Thr	Phe	Gly	Arg	Gly	Thr	Ile	Val	
			265					270					275			

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CTA TGA ACAGTTCAGT ACTAGGCAAG CCGCTGTTGG GTAAAGGCAT GTCGGAATCG 3650
 Leu *

CTGACCGGCA CACTGGATGC GCCGTTCCCC GAGTACCAGA AGCCGCTGTC CGATCCCATG 3710
 AGCGTGCTGC ACAACTGGCT OGAACGGCGA CGCCGCGTGG GCATCCGOGA ACOCGCTGOG 3770
 CTGGCGCTGG CCACGGCTGA CAGCCAGGGC CGGCCTTOGA CAOGCATCGT GGTGATCAGT 3830
 GAGATCAGTG ACACCGGGGT GCTGTTTACG ACCATGCCG GAAGCCAGAA AGGCCGCGAA 3890
 CTGACAGAGA ACCCCTGGGC CTCGGGGACG CTGTATTGGC GCGAAACCAG CCAGCAGATC 3950
 ATCCTCAATG GCCAGGCGT GCGCATGCGG GATGCCAAGG CTGACGAGGC CTGGTTGAAG 4010
 CGCCCTTATG CCACGCATCC GATGTCATCG GTGTCTCGCC AGAGTGAAGA ACTCAAGGAT 4070
 GTTCAAGCCA TCGCAACGC CGCCAGGGAA CTGGCCGAGG TTCAAGGTCC GCTGCGCGCT 4130
 CCCGAGGGTT ATTGCGTGTT TGAGTTACGG CTTGAATCGC TGGAGTTCTG GGGTAACGGC 4190
 GAGGAGCGCC TGCATGAACG CTTGOGCTAT GACCGCAGCG CTGAAGGCTG GAAACATCGC 4250
 CGGTTACAGC CATAGGGTCC CGCGATAAAC ATGCTTTGAA GTGCCTGGCT GCTCCAGCTT 4310
 CGAACTCATT GCGCAAACCT CAACACTTAT GACACCGGT CAACATGAGA AAAGTCCAGA 4370
 TCGGAAAGAA CGGTATTGCG AAATACCAAA CAGAGAGTCC GGATCACCAA AGTGTGTAAC 4430
 GACATTAACCT CCTATCTGAA TTTTATAGTT GCTCTAGAAC GTTGTCCCTG ACCCAGOGAT 4490
 AGACATCGGG CCAGAACCTA CATAAACAAA GTCAGACATT ACTGAGGCTG CTACCATGCT 4550
 AGATTTTCAA AACAAGCGTA AATATCTGAA AAGTGCAGAA TCCTTCAAAG CTT 4603

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 456 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

Met Thr Gly Ile Pro Ser Ile Val Pro Tyr Ala Leu Pro Thr Asn Arg
 1 5 10 15

Asp Leu Pro Val Asn Leu Ala Gln Trp Ser Ile Asp Pro Glu Arg Ala
 20 25 30

Val Leu Leu Val His Asp Met Gln Arg Tyr Phe Leu Arg Pro Leu Pro

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35	40	45
Asp Ala Leu Arg Asp Glu Val Val Ser Asn Ala Ala Arg Ile Arg Gln 50 55 60		
Trp Ala Ala Asp Asn Gly Val Pro Val Ala Tyr Thr Ala Gln Pro Gly 65 70 75 80		
Ser Met Ser Glu Glu Gln Arg Gly Leu Leu Lys Asp Phe Trp Gly Pro 85 90 95		
Gly Met Lys Ala Ser Pro Ala Asp Arg Glu Val Val Gly Ala Leu Thr 100 105 110		
Pro Lys Pro Gly Asp Trp Leu Leu Thr Lys Trp Arg Tyr Ser Ala Phe 115 120 125		
Phe Asn Ser Asp Leu Leu Glu Arg Met Arg Ala Asn Gly Arg Asp Gln 130 135 140		
Leu Ile Leu Cys Gly Val Tyr Ala His Val Gly Val Leu Ile Ser Thr 145 150 155 160		
Val Asp Ala Tyr Ser Asn Asp Ile Gln Pro Phe Leu Val Ala Asp Ala 165 170 175		
Ile Ala Asp Phe Ser Lys Glu His His Trp Met Pro Ser Asn Thr Pro 180 185 190		
Pro Ala Val Ala Pro Cys His His His Arg Arg Gly Gly Ala Met Ser 195 200 205		
Gln Thr Ala Ala His Leu Met Glu Arg Ile Leu Gln Pro Ala Pro Glu 210 215 220		
Pro Phe Ala Leu Leu Tyr Arg Pro Glu Ser Ser Gly Pro Gly Leu Leu 225 230 235 240		
Asp Val Leu Ile Gly Glu Met Ser Glu Pro Gln Val Leu Ala Asp Ile 245 250 255		
Asp Leu Pro Ala Thr Ser Ile Gly Ala Pro Arg Leu Asp Val Leu Ala 260 265 270		
Leu Ile Pro Tyr Arg Gln Ile Ala Glu Arg Gly Phe Glu Ala Val Asp 275 280 285		
Asp Glu Ser Pro Leu Leu Ala Met Asn Ile Thr Glu Gln Gln Ser Ile 290 295 300		
Ser Ile Glu Arg Leu Leu Gly Met Leu Pro Asn Val Pro Ile Gln Leu 305 310 315 320		
Asn Ser Glu Arg Ph Asp Leu Ser Asp Ala Ser Tyr Ala Glu Ile Val 325 330 335		

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Ser Gln Val Ile Ala Asn Glu Ile Gly Ser Gly Glu Gly Ala Asn Phe
 340 345 350
 Val Ile Lys Arg Thr Phe Leu Ala Glu Ile Ser Glu Tyr Gly Pro Ala
 355 360 365
 Ser Ala Leu Ser Phe Phe Arg His Leu Leu Glu Arg Glu Lys Gly Ala
 370 375 380
 Tyr Trp Thr Phe Ile Ile His Thr Gly Ser Arg Thr Phe Val Gly Ala
 385 390 395 400
 Ser Pro Glu Arg His Ile Ser Ile Lys Asp Gly Leu Ser Val Met Asn
 405 410 415
 Pro Ile Ser Gly Thr Tyr Arg Tyr Pro Pro Ala Gly Pro Asn Leu Ser
 420 425 430
 Glu Val Met Asp Phe Leu Ala Asp Arg Lys Glu Ala Asp Glu Leu Tyr
 435 440 445
 Met Val Val Asp Glu Glu Leu *
 450 455

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 388 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

Met Met Ala Arg Ile Cys Glu Asp Gly Gly His Val Leu Gly Pro Tyr
 1 5 10 15
 Leu Lys Glu Met Ala His Leu Ala His Thr Glu Tyr Phe Ile Glu Gly
 20 25 30
 Lys Thr His Arg Asp Val Arg Glu Ile Leu Arg Glu Thr Leu Phe Ala
 35 40 45
 Pro Thr Val Thr Gly Ser Pro Leu Glu Ser Ala Cys Arg Val Ile Gln
 50 55 60
 Arg Tyr Xaa Pro Gln Gly Arg Ala Tyr Tyr Ser Gly Met Ala Ala Leu
 65 70 75 80
 Ile Gly Ser Asp Gly Lys Gly Gly Arg Ser Leu Asp Ser Ala Ile Leu
 85 90 95

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Ile Arg Thr Ala Asp Ile Asp Asn Ser Gly Glu Val Arg Ile Ser Val
 100 105 110
 Gly Ser Thr Ile Val Arg His Ser Asp Pro Met Thr Glu Ala Ala Glu
 115 120 125
 Ser Arg Ala Lys Ala Thr Gly Leu Ile Ser Ala Leu Lys Asn Gln Ala
 130 135 140
 Pro Ser Arg Phe Gly Asn His Leu Gln Val Arg Ala Ala Leu Ala Ser
 145 150 155 160
 Arg Asn Ala Tyr Val Ser Asp Phe Trp Leu Met Asp Ser Gln Gln Arg
 165 170 175
 Glu Gln Ile Gln Ala Asp Phe Ser Gly Arg Gln Val Leu Ile Val Asp
 180 185 190
 Ala Glu Asp Thr Phe Thr Ser Met Ile Ala Lys Gln Leu Arg Ala Leu
 195 200 205
 Gly Leu Val Val Thr Val Cys Ser Phe Ser Asp Glu Tyr Ser Phe Glu
 210 215 220
 Gly Tyr Asp Leu Val Ile Met Gly Pro Gly Pro Gly Asn Pro Ser Glu
 225 230 235 240
 Val Gln Gln Pro Lys Ile Asn His Leu His Val Ala Ile Arg Ser Leu
 245 250 255
 Leu Ser Gln Gln Arg Pro Phe Leu Ala Val Cys Leu Ser His Gln Val
 260 265 270
 Leu Ser Leu Cys Leu Gly Leu Glu Leu Gln Arg Lys Ala Ile Pro Asn
 275 280 285
 Gln Gly Val Gln Lys Gln Ile Asp Leu Phe Gly Asn Val Glu Arg Val
 290 295 300
 Gly Phe Tyr Asn Thr Phe Ala Ala Gln Ser Ser Ser Asp Arg Leu Asp
 305 310 315 320
 Ile Asp Gly Ile Gly Thr Val Glu Ile Ser Arg Asp Ser Glu Thr Gly
 325 330 335
 Glu Val His Ala Leu Arg Gly Pro Ser Phe Ala Ser Met Gln Phe His
 340 345 350
 Ala Glu Ser Leu Leu Thr Gln Glu Gly Pro Arg Ile Ile Ala Asp Leu
 355 360 365
 Leu Arg His Ala Leu Ile His Thr Pro Val Glu Asn Asn Ala Ser Ala
 370 375 380
 Ala Gly Arg *

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(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 279 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

```

Met His His Tyr Val Ile Ile Asp Ala Phe Ala Ser Val Pro Leu Glu
 1             5             10             15

Gly Asn Pro Val Ala Val Phe Phe Asp Ala Asp Asp Leu Ser Ala Glu
          20             25             30

Gln Met Gln Arg Ile Ala Arg Glu Met Asn Leu Ser Glu Thr Thr Phe
          35             40             45

Val Leu Lys Pro Arg Asn Cys Gly Asp Ala Leu Ile Arg Ile Phe Thr
 50             55             60

Pro Val Asn Glu Leu Pro Phe Ala Gly His Pro Leu Leu Gly Thr Asp
 65             70             75             80

Ile Ala Leu Gly Ala Arg Thr Asp Asn His Arg Leu Phe Leu Glu Thr
          85             90             95

Gln Met Gly Thr Ile Ala Phe Glu Leu Glu Arg Gln Asn Gly Ser Val
          100            105            110

Ile Ala Ala Ser Met Asp Gln Pro Ile Pro Thr Trp Thr Ala Leu Gly
 115            120            125

Arg Asp Ala Glu Leu Leu Lys Ala Leu Gly Ile Ser Asp Ser Thr Phe
 130            135            140

Pro Ile Glu Ile Tyr His Asn Gly Pro Arg His Val Phe Val Gly Leu
 145            150            155            160

Pro Ser Ile Ala Ala Leu Ser Ala Leu His Pro Asp His Arg Ala Leu
          165            170            175

Tyr Ser Phe His Asp Met Ala Ile Asn Cys Phe Ala Gly Ala Gly Arg
 180            185            190

Arg Trp Arg Ser Arg Met Phe Ser Pro Ala Tyr Gly Val Val Glu Asp
 195            200            205

Ala Xaa Thr Gly Ser Ala Ala Gly Pro Leu Ala Ile His Leu Ala Arg
 210            215            220

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His Gly Gln Ile Glu Phe Gly Gln Gln Ile Glu Ile Leu Gln Gly Val
 225 230 235 240

Glu Ile Gly Arg Pro Ser Leu Met Phe Ala Arg Ala Glu Gly Arg Ala
 245 250 255

Asp Gln Leu Thr Arg Val Glu Val Ser Gly Asn Gly Ile Thr Phe Gly
 260 265 270

Arg Gly Thr Ile Val Leu *

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1007 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..669
- (D) OTHER INFORMATION: /gene= "phz4"
 /label= ORF4
 /note= "This DNA sequence is repeated from SEQ ID
 NO:17 so that the overlapping ORF4 may be
 separately translated"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

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Met Asn Ser Ser Val Leu Gly Lys Pro Leu Leu Gly Lys Gly Met Ser	
1 5 10 15	
GAA TCG CTG ACC GGC ACA CTG GAT GCG CCG TTC CCC GAG TAC CAG AAG	96
Glu Ser Leu Thr Gly Thr Leu Asp Ala Pro Phe Pro Glu Tyr Gln Lys	
20 25 30	
CCG CCT GCC GAT CCC ATG AGC GTG CTG CAC AAC TGG CTC GAA CGC GCA	144
Pro Pro Ala Asp Pro Met Ser Val Leu His Asn Trp Leu Glu Arg Ala	
35 40 45	
CGC CGC GTG GGC ATC CGC GAA CCC CGT GCG CTG GCG CTG GCC ACG GCT	192
Arg Arg Val Gly Ile Arg Glu Pro Arg Ala Leu Ala Leu Ala Thr Ala	

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50	55	60	
GAC AGC CAG GGC OGG CCT TCG ACA CGC ATC GTG GTG ATC AGT GAG ATC			240
Asp Ser Gln Gly Arg Pro Ser Thr Arg Ile Val Val Ile Ser Glu Ile			
65 70 75 80			
AGT GAC ACC GGG GTG CTG TTC AGC ACC CAT GCC GGA AGC CAG AAA GGC			288
Ser Asp Thr Gly Val Leu Phe Ser Thr His Ala Gly Ser Gln Lys Gly			
85 90 95			
CGC GAA CTG ACA GAG AAC CCC TGG GCC TCG GGG ACG CTG TAT TGG CGC			336
Arg Glu Leu Thr Glu Asn Pro Trp Ala Ser Gly Thr Leu Tyr Trp Arg			
100 105 110			
GAA ACC AGC CAG CAG ATC ATC CTC AAT GGC CAG GCC GTG CGC ATG CCG			384
Glu Thr Ser Gln Gln Ile Ile Leu Asn Gly Gln Ala Val Arg Met Pro			
115 120 125			
GAT GCC AAG GCT GAC GAG GCC TGG TTG AAG CGC CCT TAT GCC ACG CAT			432
Asp Ala Lys Ala Asp Glu Ala Trp Leu Lys Arg Pro Tyr Ala Thr His			
130 135 140			
CCG ATG TCA TCG GTG TCT CGC CAG AGT GAA GAA CTC AAG GAT GTT CAA			480
Pro Met Ser Ser Val Ser Arg Gln Ser Glu Glu Leu Lys Asp Val Gln			
145 150 155 160			
GCC ATG CGC AAC GCC GCC AGG GAA CTG GCC GAG GTT CAA GGT CCG CTG			528
Ala Met Arg Asn Ala Ala Arg Glu Leu Ala Glu Val Gln Gly Pro Leu			
165 170 175			
CCG CGT CCC GAG GGT TAT TGC GTG TTT GAG TTA CGG CTT GAA TCG CTG			576
Pro Arg Pro Glu Gly Tyr Cys Val Phe Glu Leu Arg Leu Glu Ser Leu			
180 185 190			
GAG TTC TGG GGT AAC GGC GAG GAG CGC CTG CAT GAA CGC TTG CGC TAT			624
Glu Phe Trp Gly Asn Gly Glu Glu Arg Leu His Glu Arg Leu Arg Tyr			
195 200 205			
GAC CGC AGC GCT GAA GGC TGG AAA CAT CGC CGG TTA CAG CCA TAGGGTCCCG			676
Asp Arg Ser Ala Glu Gly Trp Lys His Arg Arg Leu Gln Pro			
210 215 220			
CGATAAACAT GCTTTGAAGT GCCTGGCTGC TCCAGCTTCG AACTCATTGC GCAAACTTCA			736
ACACTTATGA CACCCGGTCA ACATGAGAAA AGTCCAGATG CGAAAGAACG CGTATTTCGAA			796
ATACCAAACA GAGAGTCCGG ATCACCAAAG TGTGTAAACGA CATTAACTCC TATCTGAATT			856
TTATAGTTGC TCTAGAACGT TGTCTTGAC CCAGCGATAG ACATGGGGCC AGAACCTACA			916
TAAACAAAGT CAGACATTAC TGAGGCTGCT ACCATGCTAG ATTTTCAAAA CAAGCGTAAA			976
TATCTGAAAA GTGCAGAATC CTTCAAAGCT T			1007

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(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 222 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

```

Met Asn Ser Ser Val Leu Gly Lys Pro Leu Leu Gly Lys Gly Met Ser
 1             5             10             15
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      20             25             30
Pro Pro Ala Asp Pro Met Ser Val Leu His Asn Trp Leu Glu Arg Ala
      35             40             45
Arg Arg Val Gly Ile Arg Glu Pro Arg Ala Leu Ala Leu Ala Thr Ala
      50             55             60
Asp Ser Gln Gly Arg Pro Ser Thr Arg Ile Val Val Ile Ser Glu Ile
      65             70             75             80
Ser Asp Thr Gly Val Leu Phe Ser Thr His Ala Gly Ser Gln Lys Gly
      85             90             95
Arg Glu Leu Thr Glu Asn Pro Trp Ala Ser Gly Thr Leu Tyr Trp Arg
      100            105            110
Glu Thr Ser Gln Gln Ile Ile Leu Asn Gly Gln Ala Val Arg Met Pro
      115            120            125
Asp Ala Lys Ala Asp Glu Ala Trp Leu Lys Arg Pro Tyr Ala Thr His
      130            135            140
Pro Met Ser Ser Val Ser Arg Gln Ser Glu Glu Leu Lys Asp Val Gln
      145            150            155            160
Ala Met Arg Asn Ala Ala Arg Glu Leu Ala Glu Val Gln Gly Pro Leu
      165            170            175
Pro Arg Pro Glu Gly Tyr Cys Val Phe Glu Leu Arg Leu Glu Ser Leu
      180            185            190
Glu Phe Trp Gly Asn Gly Glu Glu Arg Leu His Glu Arg Leu Arg Tyr
      195            200            205
Asp Arg Ser Ala Glu Gly Trp Lys His Arg Arg Leu Gln Pro
      210            215            220

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What is claimed is:

1. An isolated DNA molecule encoding one or more polypeptides required for the biosynthesis of an antipathogenic substance (APS) in a heterologous host, wherein said APS is selected from the group consisting of pyrrolnitrin and soraphen.
2. The isolated DNA molecule of claim 1, wherein said APS is pyrrolnitrin and said polypeptide is selected from the group consisting of SEQ ID Nos. 2-5.
3. The isolated DNA molecule of claim 1, wherein said APS is pyrrolnitrin and said DNA molecule has the sequence set forth in SEQ ID No. 1.
4. The isolated DNA molecule of claim 1, wherein said APS is soraphen and said DNA molecule has the sequence set forth in SEQ ID No. 6.
5. The DNA molecule according to any one of claims 1 to 4 engineered to form part of a plant genome.
6. An expression vector comprising the isolated DNA molecule of claim 1 wherein said vector is capable of expressing one or more polypeptides encoded by said DNA molecule in a host cell.
7. A heterologous host transformed with an expression vector comprising the isolated DNA molecule of claim 1, wherein said host is selected from the group consisting of a bacterium, a fungus, a yeast and a plant.
8. The heterologous host of claim 7, wherein said host is a plant.
9. A host capable of synthesizing an antipathogenic substance not naturally occurring in said host.
10. The host of claim 9, wherein said antipathogenic substance is selected from the group consisting of a carbohydrate containing antibiotic, a peptide antibiotic, a heterocyclic

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antibiotic containing nitrogen, a heterocyclic antibiotic containing oxygen, a heterocyclic antibiotic containing nitrogen and oxygen, a polyketide, a macrocyclic lactone, and a quinone.

11. The host of claim 10, wherein said peptide antibiotic is rhizocticin.
12. The host of claim 10, wherein said carbohydrate containing antibiotic is an aminoglycoside.
13. The host of claim 10, wherein said antipathogenic substance is a heterocyclic antibiotic containing nitrogen.
14. The host of claim 13, wherein said heterocyclic antibiotic containing nitrogen is selected from the group consisting of phenazine and pyrrolnitrin.
15. The host of claim 10, wherein said antipathogenic substance is a polyketide.
16. The host of claim 15, wherein said polyketide is soraphen.
17. The host of claim 9, wherein said antipathogenic substance is resorcinol.
18. The host of claim 9, wherein said antipathogenic substance is a methoxyacrylate.
19. The host of claim 18, wherein said methoxyacrylate is strobilurin B.
20. The host of claim 9, wherein said host is selected from the group consisting of a plant, a bacterium, a yeast and a fungus.
21. The host of claim 20, wherein said host is a plant.
22. The host of claim 21, wherein said host is a hybrid plant.

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23. Propagating material of a host according to claim 21 or 22 treated with a protectant coating.
24. Propagating material according to claim 23, comprising a preparation selected from the group consisting of herbicides, insecticides, fungicides, bactericides, nematocides, molluscicides or mixtures thereof.
25. Propagating material according to claim 23 or 24 characterized in that it consists of seed.
26. The host of claim 20, wherein said host is a biocontrol agent.
27. The host of claim 20, wherein said host is a plant colonizing organism.
28. The host of claim 20, wherein said host is suitable for producing large quantities of said APS.
29. A host capable of synthesizing enhanced amounts of an antipathogenic substance naturally occurring in said host, wherein said host is transformed with one or more DNA molecules collectively encoding the complete set of polypeptides required to synthesize said antipathogenic substance.
30. A method for protecting a plant against a phytopathogen comprising transforming said plant with one or more vectors collectively capable of expressing all of the polypeptides necessary to produce an anti-phytopathogenic substance in said plant in amounts which inhibit said phytopathogen.
31. A method for protecting a plant against a phytopathogen comprising treating said plant with a biocontrol agent transformed with one or more vectors collectively capable of expressing all of the polypeptides necessary to produce an anti-phytopathogenic substance in amounts which inhibit said phytopathogen.
32. A method for protecting a plant against a phytopathogen comprising applying to said plant a composition comprising an anti-phytopathogenic substance in amounts which inhibit

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said phytopathogen, wherein said anti-phytopathogenic substance is obtained from the host of claim 28.

33. A method for producing large quantities of an antipathogenic substance (APS) of uniform chirality comprising

- (a) transforming a host with one or more vectors collectively capable of expressing all of the polypeptides necessary to produce said APS in said host;
- (b) growing said host under conditions which allow production of said APS; and
- (c) collecting said APS from said host.

34. A composition comprising an antipathogenic substance (APS) of uniform chirality produced by the method of claim 33.

35. A method for identifying and isolating a gene from a microorganism required for the biosynthesis of an antipathogenic substance (APS), wherein the expression of said gene is under the control of a regulator of the biosynthesis of said APS, said method comprising

(a) cloning a library of genetic fragments from said microorganism into a vector adjacent to a promoterless reporter gene in a vector such that expression of said reporter gene can occur only if promoter function is provided by the cloned fragment;

(b) transforming the vectors generated from step (a) into a suitable host;

(c) identifying those transformants from step (b) which express said reporter gene only in the presence of said regulator; and

(d) identifying and isolating the DNA fragment operably linked to the genetic fragment from said microorganism present in the transformants identified in step (c);

wherein said DNA fragment isolated and identified in step (d) encodes one or more polypeptides required for the biosynthesis of said APS.

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36. An isolated polypeptide required for the biosynthesis of an antipathogenic substance (APS) in a heterologous host, wherein said APS is selected from the group consisting of pyrrolnitrin and soraphen.
37. The isolated polypeptide of claim 36, wherein said APS is pyrrolnitrin and said polypeptide is selected from the group consisting of SEQ ID Nos. 2-5.
38. The isolated polypeptide claim 36, wherein said APS is pyrrolnitrin and said polypeptide is encoded by the nucleotide sequence set forth in SEQ ID No. 1.
39. The isolated polypeptide of claim 36, wherein said APS is soraphen and said polypeptide is encoded by the nucleotide sequence set forth in SEQ ID No. 6.
40. Use of a DNA molecule according to claim 1 for genetically engineering a host organism to express said antipathogenic substance.
41. Use according to claim 40, wherein said host is selected from the group consisting of a plant, a bacterium, a yeast and a fungus.
42. Use according to claim 40, wherein the antipathogenic substance expressed does not naturally occur in said host.
43. Use according to claim 40, wherein increased amounts of the antipathogenic substance naturally occurring in said host are produced.
44. Use of the host according to claim 7 for protecting a plant against a phytopathogen.
45. Use of the composition according to claim 34 for protecting a plant against a phytopathogen.
46. Use of the DNA molecule according to claim 5 to transfer the ability to express an antipathogenic molecule from a parent plant to its progeny.

pCIB169 Restriction Map

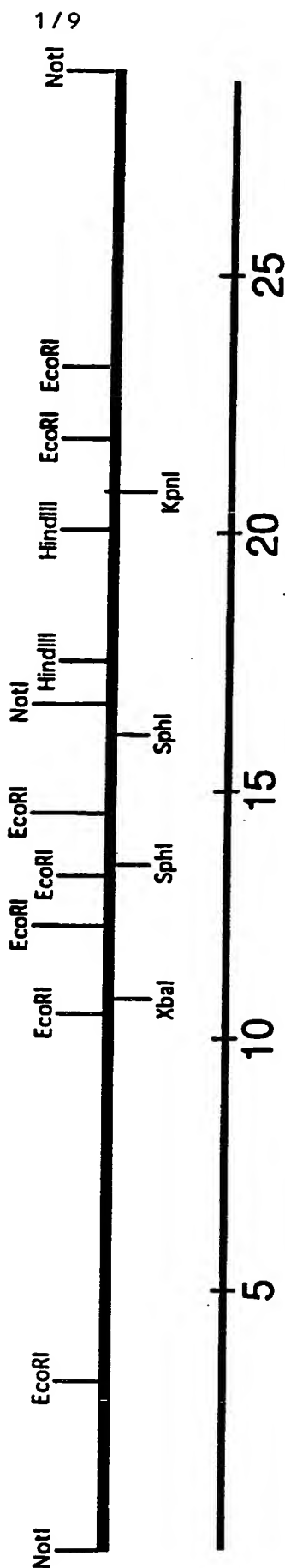


Fig. 1

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Prn Gene Region of MOCG134

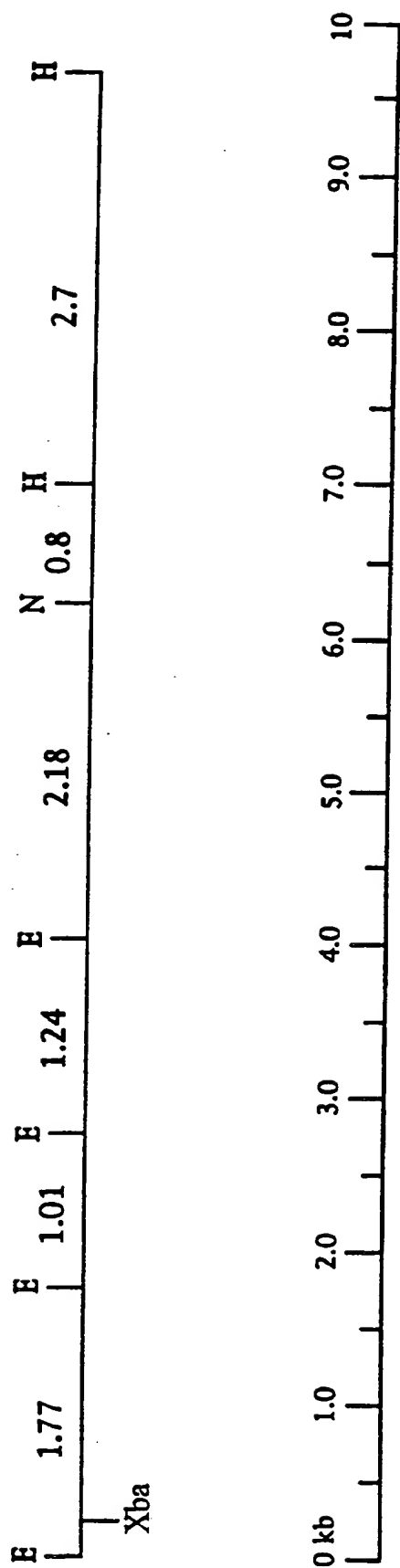


Fig. 3

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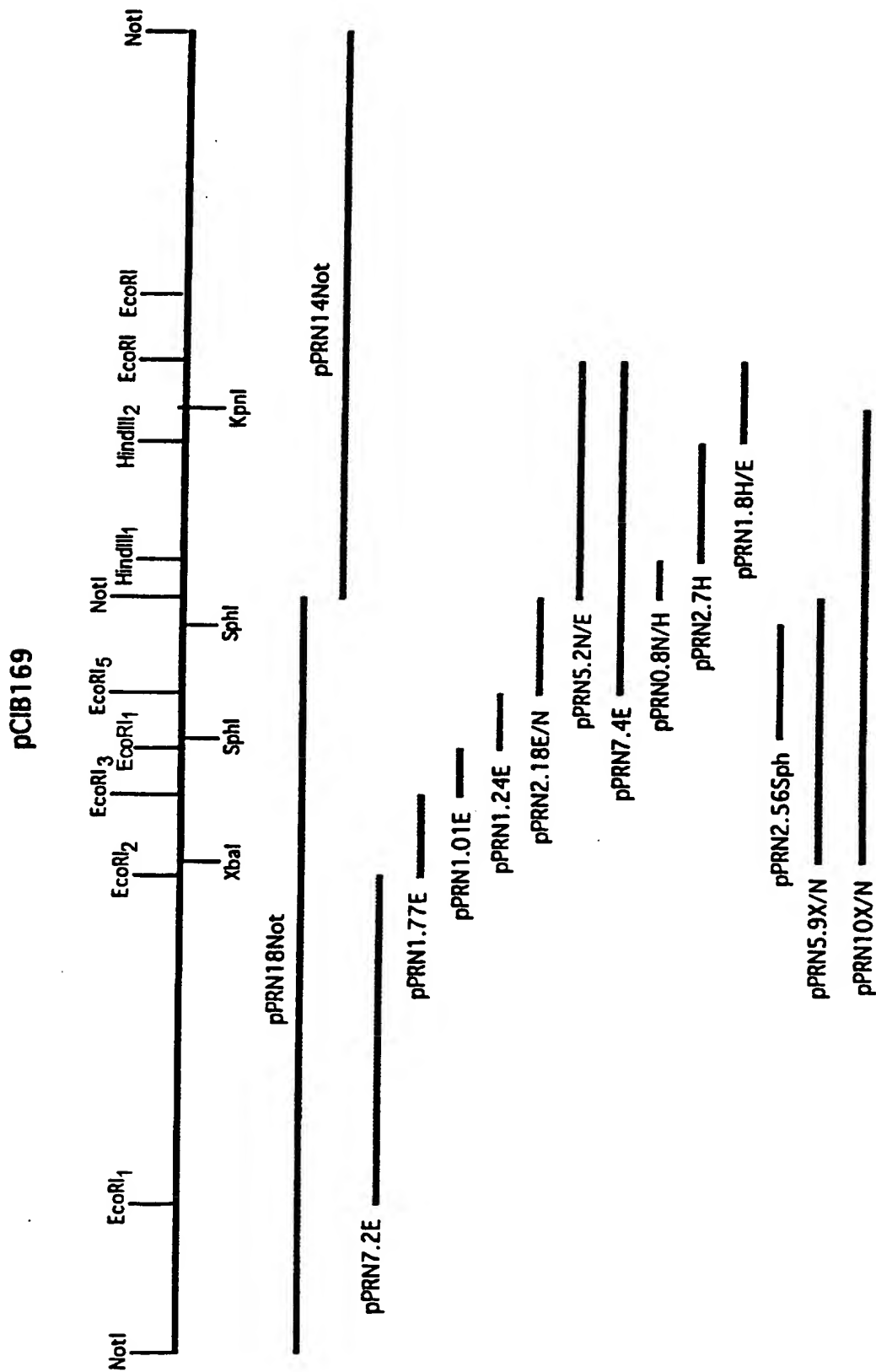


Fig. 4

Prn Gene Region of MOCG134

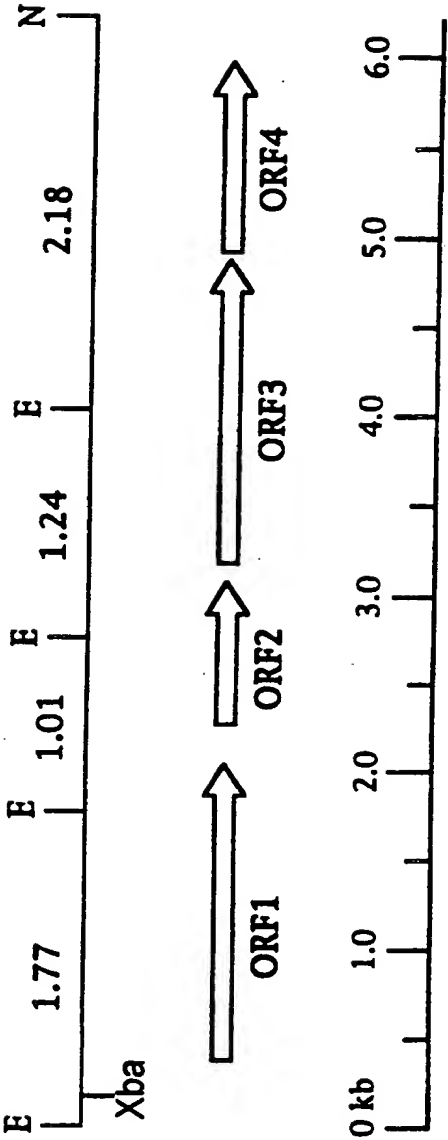


Fig. 5

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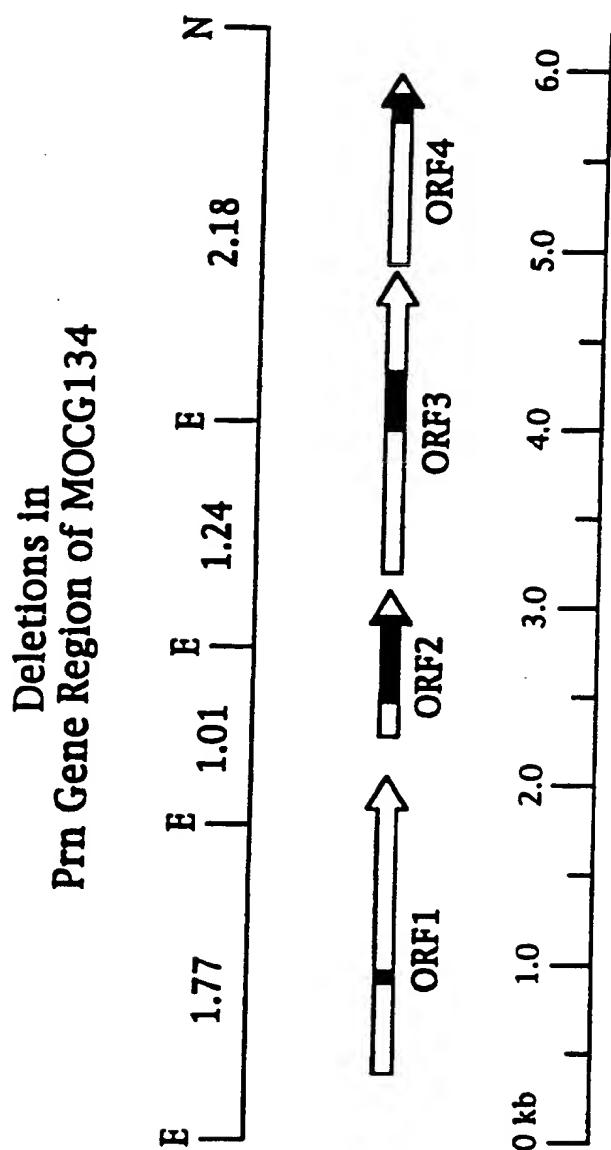


Fig. 6

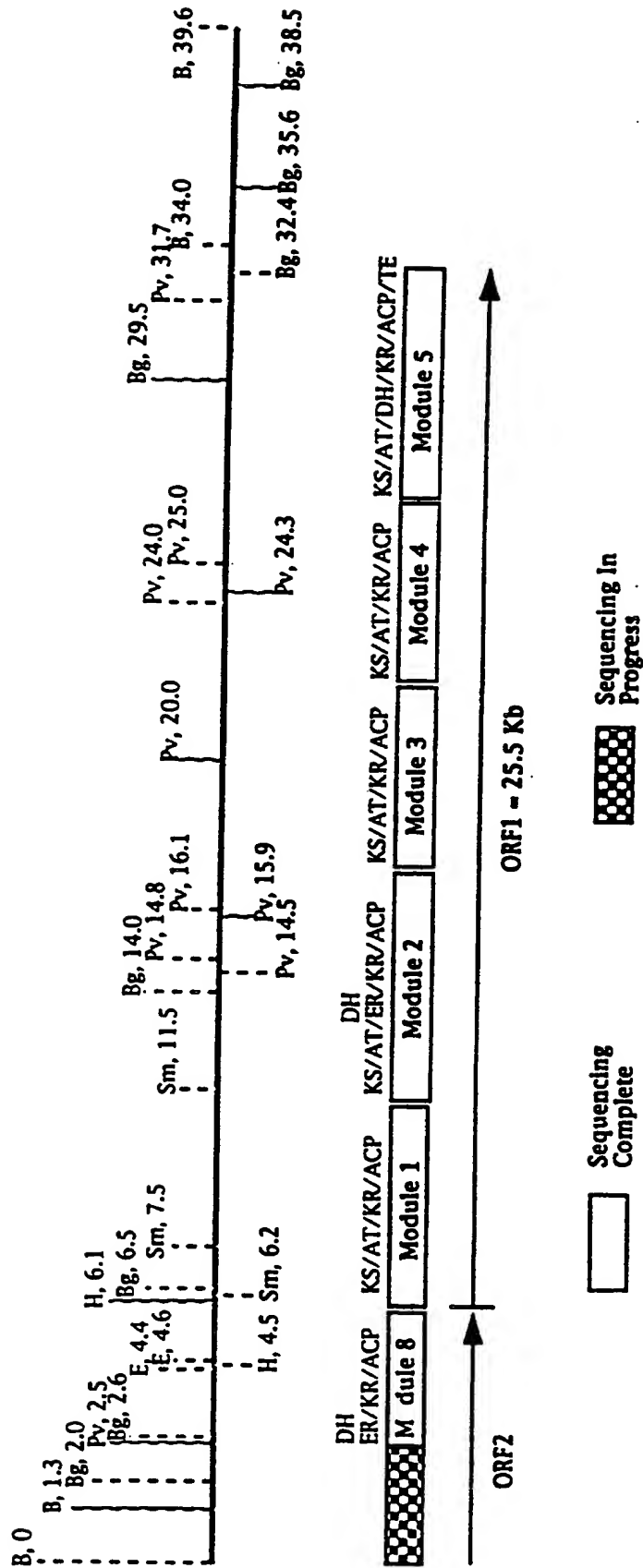


Fig. 7

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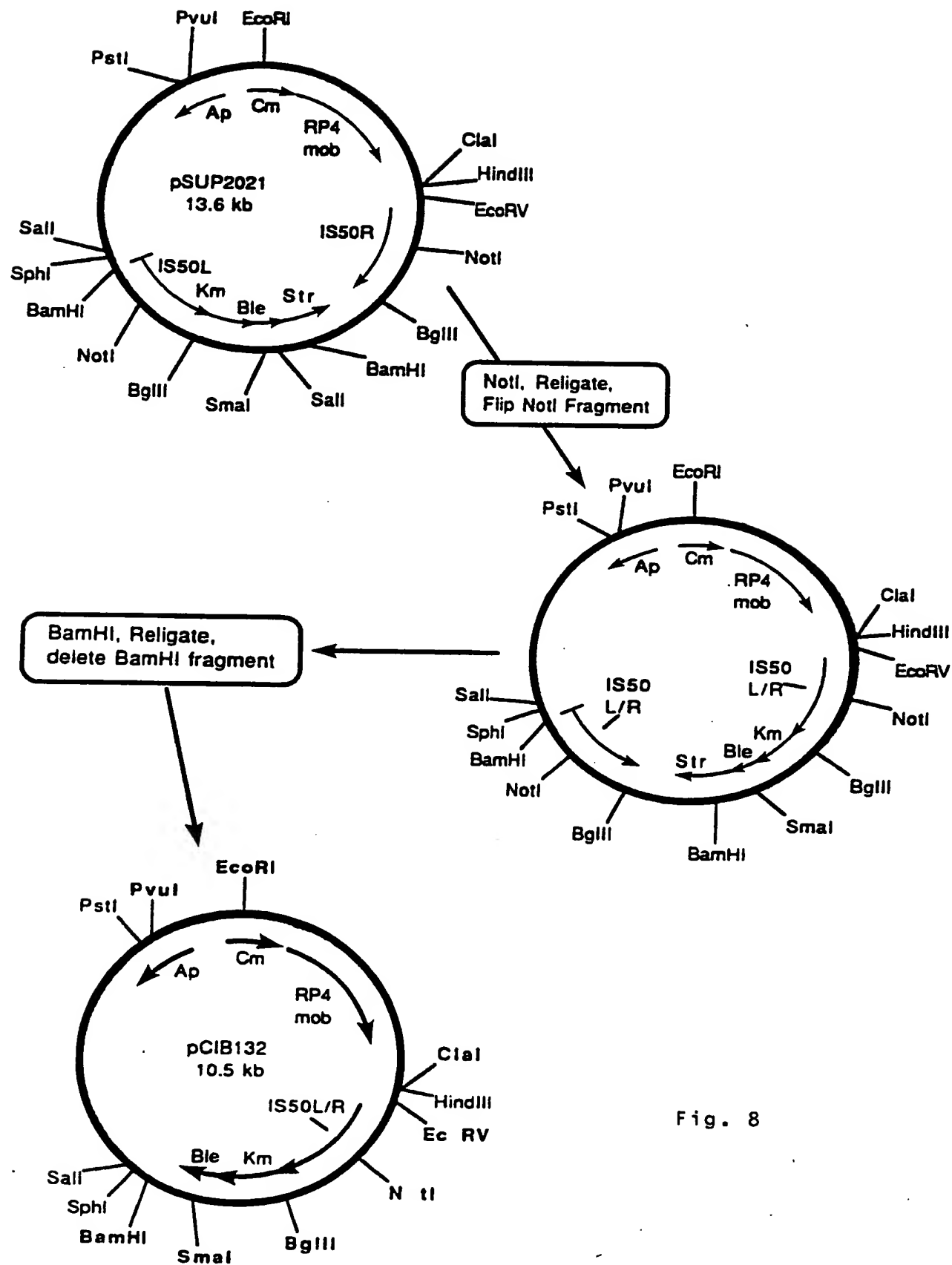


Fig. 8

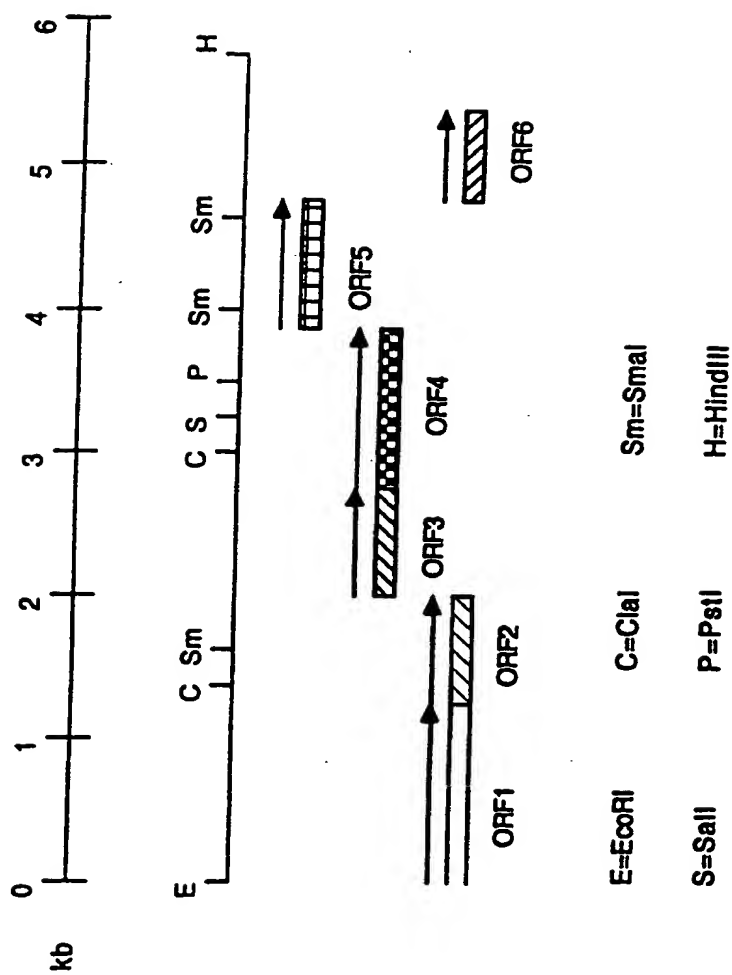


Fig. 9